THE INFLUENCE OF METHIMAZOLE AND ITS PUTATIVE METABOLITES ON HUMAN POLYMORPHONUCLEAR LEUKOCYTE FUNCTION

by

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Abbreviations

ADP	Adenosine diphosphate
AMP	adenosine monophosphate
ATP	Adenosine triphosphate
Br^-	bromine ion
C1 to C5	components of the complement system
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
c.f.u.	colony forming unit
CGD	Chronic granulomatous disease
Cl^-	chlorine ion
Cl^+	activated chlorinium ion
CMP	cytosine monophosphate
CO_2	carbon dioxide
D	Daltons
DES	diethylstilboestrol
DIT	diiodotyrosine
DMSO	dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
eg.	for example
$E\text{-}I^+$	peroxidase-iodinium complex
FAD	flavin adenine dinucleotide (oxidised form)
$Fe\ Cl_3$	ferric chloride
f-met-leu-phe N-f-mlp	formyl-methionyl-leucyl-phenylalanine
f-nle-phe-nle-tyr-lys	formyl-norleucyl-phenylalanyl-norleucyl tyrosinyl-lysine

GDH	Glasgow Dental Hospital
GDP	guanidine diphosphate
GMP	guanidine monophosphate
GRI	Glasgow Royal Infirmary
GTP	guanidine triphosphate
H^+	hydrogen ion
HBSS	Hanks basic salt solution
HBSSG	Hanks basic salt solution plus gelatin
HMPS	Hexose monophosphate shunt
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
HRPO	horse radish peroxidase
I^-	iodine ion
ie.	that is
IgA	Immunoglobulin A
IgG	Immunoglobulin G
LPO	Lactoperoxidase
M	molar
MEM	Minimal essential medium
mg	milligram
Mg^{2+}	Magnesium ion
MH	1-methylhydantoin
MI	N-methylimidazole
ml	millilitre
mm	millimetre
mM	millimolar
MMI	methimazole

$MMI^{+\cdot}$	methimazole radical cation
mol.	mole
MPO	myeloperoxidase
MTH	3-methylthiohydantoin
mV	millivolt
NaCl	sodium chloride
N.D.	not done
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaI	Sodium iodide
ng	nanogram
nM	nanomolar
NaOH	sodium hydroxide
N.S.	not significant
$^1O_2$	molecular oxygen
O_2	singlet oxygen
O_2^-	superoxide
OH•	hydroxyl radical
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
PTU	propylthiouracil
RNA	ribonucleic acid
s	standard deviation
S1	first stimulus
S2	second stimulus
-SH group	sulphydryl group

SIS	stock isotonic solution
SOD	superoxide dismutase
T_3	triiodothyronine
T_4	thyroxine
TPO	thyroid peroxidase
TPO_{ox}	oxidised form of TPO
$TPO.I^+$	intermediate oxidation product between TPO and drug
$TPO.I_{ox}$	TPO-oxidised iodine complex
µg	microgram
µm	micrometre
µM	micromolar
W/V	weight/volume
$\bar{x}$	mean
C.albicans	Candida albicans
E.coli	Escherichia coli
L.acidophilus	Lactobacillus acidophilus
L.casei	Lactobacillus casei
M.lysodeikticus	Micrococcus lysodeikticus
Ps.aeruginosa	Pseudomonas aeruginosa
S.albus	Staphylococcus albus
S.aureus	Staphylococcus aureus
S.pyogenes	Streptococcus pyogenes
S.faecalis	Streptococcus faecalis

Summary

An uncommon side effect of treatment with antithyroid drugs is agranulocytosis. These compounds are also known to be capable of modifying immune function in humans. The polymorphonuclear leukocytes are responsible for clearing invading foreign bodies from the bloodstream, and as such are the first line of defence against infection. The effect of methimazole, a thioureylene antithyroid drug commonly used in the treatment of hyperthyroidism, on selected parameters of polymorphonuclear leukocyte function, was investigated. Methimazole is metabolised by polymorphonuclear leukocytes, therefore the influence of some of its putative metabolites, namely 3-methylthiohydantoin, methylhydantoin and N-methylimidazole, was also assessed. The chemotactic, phagocytic and cidal capabilities of human polymorphonuclear leukocytes were examined.

The ability of polymorphonuclear leukocytes to migrate under agarose towards the chemo-attractant, zymosan-stimulated serum, was found to be unaffected by methimazole, 3-methylthiohydantoin, methylhydantoin and methylimidazole at concentrations between 10^{-3} and 10^{-7} M. Phagocytosis of *S. aureus*, *S. pyogenes*, *E. coli*, *L. casei* and *C. albicans* proceded normally in the presence of 10^{-3} M methimazole, *S. pyogenes* and *S. aureus*, as representatives of hydrogen peroxide- and non-hydrogen peroxide producing bacteria respectively were the subject of further investigation which established that

methimazole, 3-methylthiohydantoin, methylhydantoin and N-methylimidazole did not significantly affect their ingestion at concentrations between 10^{-3} and 10^{-5} M.

Intracellular killing of all five pre-opsonised micro-organisms by human polymorphonuclear leukocytes treated with 10^{-3} M methimazole was stimulated by between ten and 40 per cent after 30 minutes co-incubation. Between 60 and 120 minutes there was no evidence of any significant stimulation. Further investigation with S. pyogenes and S. aureus revealed that methimazole, 3-methylthiohydantoin, methylhydantoin and N-methylimidazole exerted no significant effect on the killing of S. pyogenes, however dose-related inhibition was evident on treatment with between 10^{-3} and 10^{-5} M methimazole and 3-methylthiohydantoin during phagocytosis of S. aureus. Methylhydantoin and N-methyl-imidazole produced no significant effects.

The oxygen consumption of latex-stimulated polymorphonuclear leukocytes was found to be stimulated by 48 and 58 per cent respectively by methimazole and N-methylimidazole at 10^{-3} M, 3-methylthiohydantoin and methylhydantoin producing no significant change. The hexose monophosphate shunt activity of similarly stimulated polymorphonuclear leukocytes significantly increased by up to 20 per cent in the presence of methimazole, 3-methylthiohydantoin and N-methylimidazole.

None of the compounds exerted any influence on the release of lysosomal enzymes by stimulated polymorphonuclear leukocytes, however methimazole, 3-methylthiohydantoin and N-methylimidazole inhibited myeloperoxidase activity by between 36 and 100 per cent at 10^{-3} M, this inhibition was dose-related at concentrations between 10^{-3} and 10^{-5} M. Lysozyme and lactoferrin activity from the released lysosomal enzymes was unaffected by any of the four compounds, neither was the lysozyme activity of intact polymorphonuclear leukocytes. Alkaline phosphatase activity however was inhibited by around 50 per cent by methimazole and 3-methylthiohydantoin at concentrations of 10^{-3} M; again the inhibitory effect was significantly dose-related.

Thus it would appear that methimazole acts, not by inhibiting the microbicidal oxidase system, as might have been expected, but by altering myeloperoxidase activity. It may combine in some way with myeloperoxidase so that it can no longer react with hydrogen peroxide.

The killing of S. pyogenes was unaffected by methimazole, perhaps due to the many backup systems of the polymorphonuclear leukocyte, which are believed to be stimulated in polymorphonuclear leukocytes with reduced or no myeloperoxidase activity. S. aureus was killed less efficiently: this organism contains the enzyme catalase, which catalyses

the decomposition of hydrogen peroxide to water and oxygen, rendering S. aureus more resistant to attack by non-myeloperoxidase-mediated killing by hydrogen peroxide, simply by decomposing the hydrogen peroxide.

3-Methylthiohydantoin and to a lesser extent N-methylimidazole acted in a similar fashion to methimazole, except that 3-methylthiohydantoin appeared to be slightly more potent in its inhibitory action on the polymorphonuclear leukocyte, and N-methylimidazole less so. Methylhydantoin did not significantly affect polymorphonuclear leukocyte function.

Fosfestrol is a water-soluble oestrogen compound with a similar oxidation potential to methimazole. It is used in the treatment of prostatic carcinoma. It was thought that it might act on the polymorphonuclear leukocyte in a similar way to methimazole, therefore its effect on the various polymorphonuclear leukocyte functions was assessed.

Like methimazole, fosfestrol at concentrations between 10^{-3} and 10^{-5} M did not significantly affect chemotaxis, random movement or phagocytosis, neither did it possess any chemo-attractant or -repellant activity. However killing of S. aureus after 30 minutes incubation was significantly inhibited (14 per cent) by 10^{-3} M fosfestrol. Killing of S. pyogenes, E. coli, L. casei and C. albicans was not

significantly altered. After 60 and 120 minutes there was a trend towards slight stimulation of killing. C. albicans showed 12 per cent greater killing after 60 minutes with 10^{-3} M fosfestrol treatment, this stimulation being dose-related. Oxygen consumption was inhibited by 16–18 per cent and hexose monophosphate shunt activity stimulated by 18 per cent. Myeloperoxidase, alkaline phosphatase, lysozyme and lactoferrin activity was not significantly affected by up to 10^{-3} M fosfestrol. Electron microscopic examination of C. albicans within control and fosfestrol-treated polymorphonuclear leukocytes revealed slightly more degradation of Candida within fosfestrol-treated polymorphonuclear leukocytes. This was manifest as loss of striations within the thick cell wall, loss of organelles within the yeast cells, which became less electron dense and eventual detachment of the plasma membrane from the cell wall.

In a clinical situation, therefore, neither methimazole, fosfestrol, nor any of the proposed metabolites investigated in this study would be expected to exert any significant effect on the ability of an otherwise non-compromised individual to combat infection whilst undergoing treatment.

CHAPTER 1

REVIEW OF THE PROCESSES OF PHAGOCYTOSIS AND KILLING OF MICRO-ORGANISMS BY POLYMORPHONUCLEAR LEUKOCYTES, AND THE ACTION OF METHIMAZOLE ON THESE

1.0 Historical Aspects.

In 1882, the Russian zoologist Elie Metchnikoff, while studying the behaviour of mobile cells in the transparent starfish larva, noted that the introduction of a sharp thorn into the body of the larva caused the mobile cells to surround the foreign body and apparently to attack it. Moreover, these cells could also ingest and destroy foreign particles, including bacteria. Cohnhein had earlier seen white blood cells migrating from tissue capillaries to form pus at sites of injury. Metchnikoff concluded that inflammation served as an important defence reaction of the body. "In man", he wrote "microbes are usually the cause which provokes inflammation, therefore, it is against these intruders that the mobile mesodermic cells have to strive. The mobile cells must destroy the microbes by digesting them and thus bring about a cure. Inflammation, therefore, is a curative reaction in the organism and morbid symptoms are no other than the signs of the struggle between the mesodermic cells and the microbes." Metchnikoff's theory was attacked by his colleagues and a controversy arose between the followers of Metchnikoff's theory of cellular immunity and the majority of clinicians and pathologists

who adhered to the theory of humoral immunity. Both are now known to play a part in the body's defence against infection.

The humoral immune system, comprising antibody and complement, is often able to deal with invading pathogens. However, the cellular and humoral components must co-operate to destroy better-equipped pathogens by the process of phagocytosis.

One such phagocyte is the polymorphonuclear leukocyte (PMN). PMN comprise about 65% of the white blood cell population in humans and are mainly involved in defence against micro-organisms which elicit an acute inflammatory response.

Phagocytosis refers to the physical act of engulfment, the process by which particles recognised by the phagocyte as foreign are taken up by the cell and sequestered in an intra-cellular vacuole. The process can be divided into a number of phases:- i) chemotaxis, ii) recognition of foreign particles, iii) attachment of particles to the phagocyte surface and iv) ingestion of particles.

1.1 Chemotaxis.

Chemotaxis is defined as directed movement of cells or of organisms in response to a concentration gradient of a mediator. PMN are mobile cells. The accumulation of leukocytes in inflammatory sites is an important aspect of normal host defence and motility plays a central role in

this function.

Chemotaxis must be clearly distinguished from random migration. Random motility is movement which occurs with equal probability in all directions, (Miller, 1975a,b: Bandmann et al, 1974) and is an inherent property of leukocytes; however their exposure to a uniform concentration of a chemotactic agent, ie. no gradient, can result in increased random migration. (Keller and Sorkin, 1966, 1967; Gallin and Rosenthal, 1974). Chemotaxis is also distinct from chemokinesis; chemotaxis is a reaction by which the direction of locomotion is changed; chemokinesis is an alteration in the speed of mobilisation.

The acute inflammatory response involves mobilisation of leukocytes. This requires active locomotion from the blood vessels through the capillary endothelium and into the site of injury. This directed migration occurs under the influence of soluble mediators, the precise nature of which varies according to the type of injury. Microbial invasion, immunologic injury and non-specific tissue damage may all involve the formation of PMN chemotactic factors. Thus chemotaxis is the first step in the phagocytic process; the directional movement towards the material to be engulfed.

Chemotactic Factors

A large number of neutrophil chemotactic factors have been described. Historically, bacterial chemotactic factors were

recognised first, but much research has gone into chemotactic factors of host origin, such as those derived from the complement system.

Bacterial Chemotactic Factors

Bacteria frequently produce chemotactic factors that attract leukocytes directly and do not require the presence of serum. Such factors have been termed "cytotaxins". However, the direct attractive effect of bacteria is often weak, and a strong effect is seen only in the presence of serum. Factors that generate chemotactic activity by activating serum components have been termed "cytotaxigens".

The early research into chemotaxis did not distinguish between cytotaxins and cytotaxigens, as the role of complement components in chemotaxis was not appreciated.

Leber (1888) using an extract of Staphylococcus aureus as an attractant, first demonstrated chemotaxis in PMN. McCutcheon (1946) and Harris (1954) reviewed the early literature on chemotaxis, confirming that a wide variety of microorganisms release cytotaxins.

Most organisms studied up to the present time are capable of generating complement-mediated chemotactic activity (Wilkinson, 1980). Many bacterial surface structures alone are capable of this. The endotoxins of Gram-negative bacteria are examples of substances with little direct chemotactic activity, but are

powerful activators of complement. (Shin et al, 1968; Snyderman et al, 1969).

The precise nature of bacterial chemotactic factors has been studied by many investigators. Keller and Sorkin (1967) found serum-independent activity in culture filtrates of Staphylococcus albus. A chemotactic factor precipitable by ammonium sulphate was partially purified from filtrates of Staphylococcus aureus (Walker et al, 1969). Staphylococcal enterotoxin B generates chemotactic activity in the presence of serum (Craig et al, 1971). Protein A in combination with the F(c) fragments of IgG can activate complement and produce chemotactic activity (Harvey et al, 1970).

Russell et al, (1976) using chloroform-methanol-water extraction showed that some of the chemotactic activity of staphylococci was present in chloroform-soluble lipid fractions and the methanol-water fraction also contained some activity.

Ward et al (1968), found direct attractant activity in culture filtrates of alpha-haemolytic streptococci and of Streptococcus pneumoniae. The pneumococcal factor had a molecular weight of less than 3,600 and was not related to the pneumococcal polysaccharide. Streptococci contain proteases that can activate chemotactic complement peptides (Ward et al, 1973). Taylor et al (1967) and Ward (1967) independently reported that streptokinase which converts plasminogen to plasmin, generates chemotactic activity in serum by a proteolytic

action of plasmin on C3. It has since been shown that C3a and other C3 derived peptides have no chemotactic activity, (Fernandez et al, 1978) therefore their conclusions probably require reassessment.

Culture filtrates of Escherichia coli have probably been the most extensively studied of all bacterial chemotactic factors, and are used by many as standard stimuli in chemotaxis assays. Trainer et al (1975) found chemotactic activity in chloroform-methanol extracts of E.coli that had fatty acid-like properties. Sahu and Lynn (1977) found around 75% of the total chemotactic activity in the filtrates they examined was due to lipids. Schiffman et al (1975) extracted chemotactically active peptides with molecular weights of 150-1,500 from filtrates of E.coli. These peptides were anionic, had blocked N-termini and were heterogeneous. These findings led to the discovery of the formyl-methionine peptides, which are probably the most widely used chemotactic factors at the present time. E.coli initiates the sequence of events involved in protein synthesis with N-formyl-methionine. This initiating amino acid is cleaved from the newly synthesised protein before its release. Since eukaryotic cells do not commence protein synthesis with formyl-methionine, except in mitochondria, the initiating sequence in prokaryotic cells could be a convenient signal that allows phagocytic cells to recognise invading pathogens as foreign. Marasco et al (1983) identified and purified formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe) as the major peptide neutrophil chemotactic

factor produced by E.coli.

Schultz and Miller (1974) showed an elastase produced by Pseudomonas aeruginosa could cleave a number of complement components and caused release of C5a from C5 when used at low doses, but destroyed the peptide at high doses.

Candida albicans, a yeast, is also known to generate chemotactic activity in the presence of complement (Cutler, 1977). Ofek and Bekierkunst (1976) have reported direct chemotactic activity generated by mycobacterial cord factor (trehalose 6,6-dimycolate) for macrophages and PMN.

Thus most bacteria produce or induce chemotactic factor(s), the nature of which is diverse.

Complement-derived Chemotactic Factors

Boyden (1962) observed that certain serum components could generate PMN leukocyte chemotactic factors. He found that incubation of mixtures of antigen and specific antibody with fresh serum resulted in the appearance of chemotactic activity. Preheating the serum at 56°C for 30 minutes destroyed the activity, but once the factor was produced, it was heat stable. Similar findings were reported by Keller and Sorkin (1965). Boyden interpretted the results as "antibody and antigen combine to form a complex which interacts with a heat-labile substance (probably an enzyme, perhaps a component of complement) and that as a consequence of this interaction a heat-

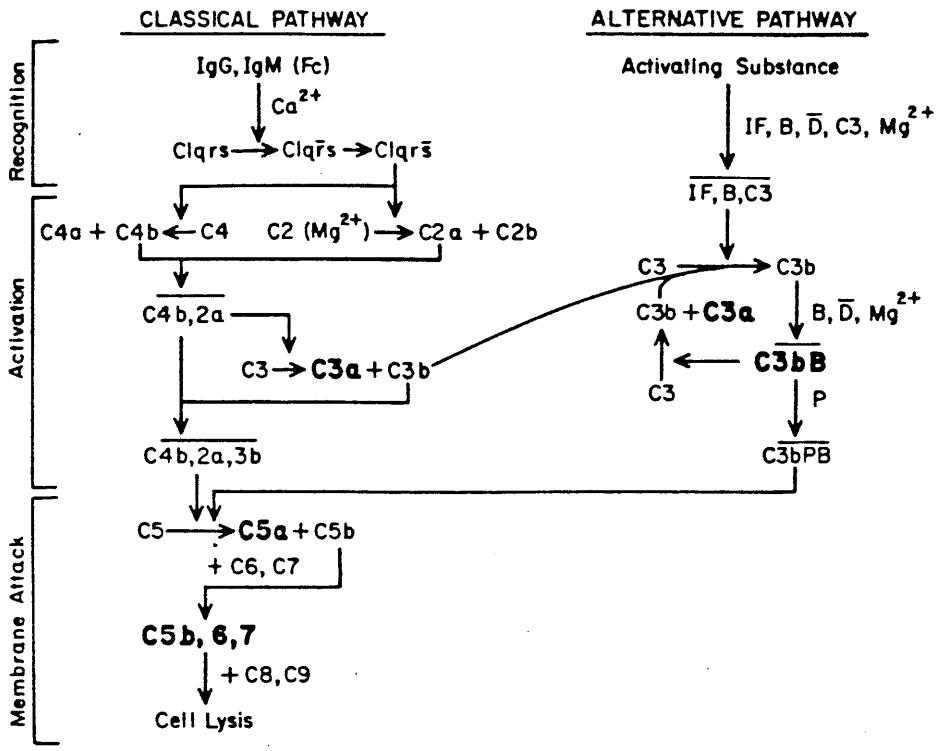


Figure 1. Proposed pathways of complement activation. Agents with chemotactic activity are indicated in bold face. A bar above a component indicates an enzymatically active protein.

stable substance is produced which has a direct chemotactic influence on the leukocytes". Subsequent studies fully supported these conclusions.

Complement refers to a series of serum proteins which are activated sequentially and function as a mechanism for the destruction of foreign cells and the production of a number of biologically active mediators. Two pathways of complement activation have been described: the classical pathway and the alternative (properdin) pathway (Fig 1).

Activation of the complement system via the classical pathway or the alternative pathway releases a number of chemotactically active compounds. The most important of these is C5a, which accounts for most of the chemotactic activity produced on complement activation.

Human C5a is a glycoprotein which contains a polypeptide portion of 74 amino acids which has been sequenced, accounting for a molecular weight of 8,200, and a carbohydrate portion accounting for 3,000 Dalton (Fernandez and Hugli, 1976). C5a is chemotactically active in doses as low as 1.0nM. The most prevalent form of human C5a found *in vivo* is $C5a_{desarg}$, a molecule from which the COOH- terminal arginine has been removed by endogenous carboxypeptidase enzymes (Fernandez *et al*, 1978).

C5a exhibits classical anaphylotoxic activity; it contracts

smooth muscle and degranulates mast cells (Shin et al, 1968; Jensen et al, 1969). It has been identified at sites of experimental inflammation in vivo (Snyderman et al, 1971) and also in the synovial effusions of patients with inflammatory arthritis (Ward and Zvaifler, 1971).

PMN Leukocyte-derived Chemotactic Factors

Evidence of chemotactic properties of a number of other agents have been published, these include a cell-derived chemotactic factor produced by PMN that have ingested crystalline material such as monosodium urate or calcium pyrophosphate dihydrate (Spilberg and Mehta, 1979; Spilberg et al, 1976). Products of arachidonic acid lipo-oxygenation have also been shown to be chemoattractants (Goetzl and Pickett, 1980; Turner et al, 1975). Leukotriene B_4 is the most potent chemo-attractant of arachidonic acid metabolism (Ford-Hutchinson et al, 1980, 1981).

The Cellular Chemotactic Response

Detection Of The Chemotactic Stimulus

Contact between the chemotactic factor and the phagocyte cell surface results in activation of receptor sites which initiates movement. The directional character of the response could result from one of two mechanisms. A single receptor unit might sense a concentration of a chemotactic factor at one point and then, after migration in some arbitrary direction, sense the concentration again, comparing the new level with the previous one to determine the directionality of the concentration gradient. Alternatively, multiple receptor sites

might simultaneously assess concentrations at various locations on the cell and detect a gradient of chemotactic factor across the cell's own dimensions. Support for the latter has been gained from the observations of Ramsey (1972a, b) and Zigmond (1974). Their direct observations of single migrating PMN demonstrate that the initial direction of migration, immediately after exposure to a chemo-attractant, is towards the stimulus in the majority of instances. Movement of the stimulus after migration has begun results in a rapid change in direction of migration towards the new stimulus location. Migration consists of a series of short, straight movements separated by relatively sharp turns, such that the direction and magnitude of each turn tends on the average to bring the cell closer to the stimulus. Nossal and Zigmond (1976) have developed a mathematical model characterising the movement of individual cells as related to the turn-angle probability distributions; experimental observations suggested that both the direction of turning and the turn-angle distribution was modified by a chemotactic gradient. This data is consistent with the sensing of a spatial gradient of the attractant, and monitoring of the direction of migration by continuous sensing of the attractant gradient. Under most circumstances, the concentration difference of a diffusing chemo-attractant across the diameter of a neutrophil (up to 16μm) would be extremely small, necessitating very precise sensing mechanisms. PMN are known to respond to as little as a 1% gradient of formyl peptide across their length (Zigmond and Sullivan, 1979). Seligman et al (1982) have suggested that decreases in f-met-leu-phe

receptor affinity, "through either heterogeneity or negative co-operativity", is important in neutrophil adaptation and could allow the cell to sense very small changes in attractant concentration when the ambient peptide concentration was low (conditions required for sensing a gradient during directed locomotion) and still respond at high peptide concentrations with degranulation and respiratory burst activities (conditions expected when cells have arrived at inflammatory foci).

These proposals have been investigated and experimental data demonstrate that the optimal chemotactic concentration of f-met-leu-phe is one to twofold lower than that for secretion or superoxide production (Lohr and Snydermen, 1982). It appears that adaptation represents a decrease in the affinity of neutrophil responsiveness to f-met-leu-phe, with no effect on the maximal responsiveness of cells so long as the initial stimulus concentration is less than 10^{-8} M. At concentrations greater than 10^{-8} M, not only does the apparent affinity decrease but the maximal responsiveness of the cells decreases.

Studies of the dependence of the second (S2) stimulus on the first stimulus (S1) concentration indicated that with S1 concentrations below 10^{-8} M, small increases in S2 were needed to elicit responses. However with S1 concentrations greater than 10^{-8} M, large increases in S2 were required to give a later response (Gallin et al, 1983). Gallin and Seligman (1984) using information from these studies and the observation that it takes about one and a half minutes for cells to adapt

to a chemo-attractant stimulus, predicted the rate at which cells would have to move in a gradient of chemo-attractant to keep up with, or just ahead of, the change in receptor affinity as they move up a gradient of stimulus. When cells are exposed to concentrations of f-met-leu-phe of less than 10^{-8} M the predicted rate of locomotion would be 3-10 μm/min, compatible with measured rates. With f-met-leu-phe concentrations of greater than 10^{-8} M, the predicted rate of cell migration would be much faster, 30-40 μm/min, to remain equivalently stimulated, rates which PMN cannot achieve. Hence, the cells would stop moving at the higher concentrations of chemo-attractant.

This progressive modulation of receptor affinity after stimulation may be initially important for sustaining chemotaxis and later for converting cells whose predominant function is chemotaxis, into cells whose predominant function is degranulation and production of highly reactive products of oxidative metabolism.

It has been proposed that there exists within PMN a pool of receptors, which are expressed at the cell surface after stimulation. Gallin et al (1984) have proposed the idea of receptor recycling. After f-met-leu-phe interaction with its receptor on PMN, there is internalisation of bound ligand, with down regulation of receptor expression (Niedel et al, 1979; Zigmond and Sullivan, 1979; Donabedian and Gallin, 1981; Sullivan and Zigmond, 1982). Later the receptors

reappear. There is no direct evidence for receptor recycling, but the final steady state of f-met-leu-phe receptor expression exceeds prestimulation levels (Fletcher and Gallin, 1980; Fletcher et al, 1982). Therefore, either synthesis of a new pool of receptors, or mobilisation of an intracellular compartment of receptors must exist. Gallin et al (1979) proposed that the new receptors come from specific granules or other organelles such as Golgi vesicles, as increases in the number of receptors occurs in the absence of protein synthesis. Jesaitis et al (1982) also proposed a spare pool of f-met-leu-phe receptors in Golgi vesicles.

The expression of these receptors and the addition of new membrane may be critical in the localisation of cells at specific sites in the circulation, and for the transduction of signals to launch such processes as chemotaxis, degranulation, oxidative metabolism; phagocytosis and destruction of micro-organisms.

The Nature Of Chemo-attractant Receptors

The identification of a series of formylated peptides with potent chemotactic activity eg f-met-leu-phe, has made possible the investigation of receptor sites on PMN. The strict structure-function relationships of synthetic N-formylated oligopeptides for initiating chemotactic and secretory activities led to the suggestion that leukocytes recognised chemo-attractants via receptors (Showell et al, 1976; Freer et al, 1980). Williams et al (1977), using radio-labelled f-met-leu-phe showed that PMN possessed receptors and that binding was

saturable, the number of binding sites per cell ranged from 40,000-60,000. Niedel et al (1979), demonstrated receptors for f-nle-phe-nle-tyr-lys and estimated 120,000 receptors per human PMN.

Niedel et al (1980) attempted to characterise chemo-attractant receptors biochemically. He found the receptor for f-nle-phe-nle-tyr-lys to be a polypeptide with an apparent molecular weight of 55,000-70,000. Schmitt et al (1983) using photo-affinity labelling to investigate the N-formylated peptide receptor, found a polypeptide with molecular weight of 50,000-60,000 Daltons.

Signalling Mechanisms Within The Cell

The affinity of the chemo-attractant receptor is heterogeneous and dynamically regulated both by guanine nucleotides and prior agonist exposure (Snyderman and Pike, 1984).

Transduction of chemo-attractant-related signals requires transmethylation reactions mediated by S-adenosylmethionine and leads to the activation of phospholipases that liberate arachidonic acid from membrane phospholipids. Recent evidence suggests that protein kinase C is also involved in the transduction of some chemo-attractant-induced signals (Hirata et al, 1979; Snyderman et al, 1980; McPhail et al, 1984).

Snyderman and Pike (1984) found that the effect of the non-hydrolysable analogue of GTP (p(NH)ppG), was to convert

f-met-leu-phe receptors originally present in the high affinity state to a low affinity state. This effect was also brought about by GTP and GDP, however ATP, ADP, AMP, cAMP, GMP and CMP had no effect.

It is known that chemo-attractants have the ability to regulate the affinity of the receptors to which they bind. Koo and Snyderman (1983) showed an increase in the percentage of high-affinity receptors expressed on stimulation of human PMN membranes with f-met-leu-phe. Snyderman and Pike (1984) have suggested that the high-affinity site might be an immediate form of the receptor which triggers internalisation or perhaps transduction of a signal for secretion.

Changes in membrane lipids also appear to be required for chemo-attractant-mediated functions. Rapid release of arachidonic acid from membrane phospholipids in PMN occurs following chemo-attractant receptor occupancy (Hirata et al, 1979; Pike and Snyderman, 1981). The free arachidonic acid is then available for synthesis of prostaglandins, thromboxanes, prostacyclins and leukotrienes, all of which are potent mediators of inflammatory and vasomotor responses. Free arachidonic acid itself may be an important second messenger of chemo-attractant-mediated responses (McPhail and Snyderman, 1984). It has been suggested that chemoattractants mobilize arachidonic acid via a phospholipase C activation (Pike and Snyderman, 1981).

Over the past few years, studies have shown that transmethylation reactions mediated by S-adenosyl-methionine play an important role in the chemotactic response of leukocytes (O'Dea et al, 1978; Pike et al, 1978). Methylation reactions are also required for other chemo-attractant-mediated functions in mononuclear cells, such as superoxide production and arachidonic acid release from phospholipids (Pike and Snyderman, 1981; 1982). Inhibition of methylation lowers the receptor's affinity by producing an allosteric alteration in the receptor.

A phospholipid- and calcium-dependent protein kinase (protein kinase C) has been identified (Castagna et al, 1982) and found to be widely distributed in tissues. The activity of this enzyme is enhanced by diolein, a substance formed through the action of phospholipase C on membrane phospholipids. It has been shown that chemo-attractants do not activate adenylate cyclase in leukocyte membranes (Verghese and Snyderman, 1983) and from this it has been reasoned that protein kinase C may mediate certain chemo-attractant-receptor functions. Snyderman and Pike (1984) have found that human PMN contain substantial protein kinase activity. Under conditions that initiate secretion and the respiratory burst, f-met-leu-phe also produces translocation of the enzyme from the cytosolic fraction of cells to the particulate fraction.

Arachidonic acid, as well as other unsaturated long-chain fatty acids, are capable of activating leukocyte protein kinase C directly in leukocyte cytosolic fractions in the

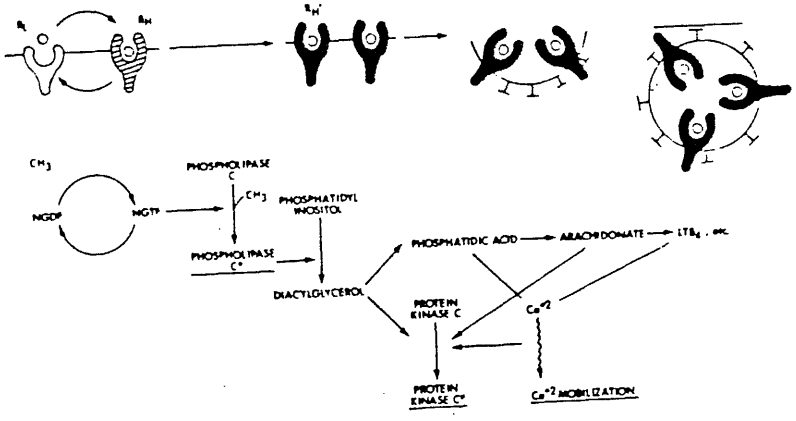


Figure 2. Model of chemoattractant receptor transduction. It is hypothesized that binding of the chemoattractant to its receptor leads to the formation of a reversible high-affinity state (hatched line receptor) regulated by guanine nucleotides. At high concentrations of the chemoattractant, a nonnucleotide-sensitive high-affinity receptor is formed (blackened receptor). This receptor may be rapidly internalized, perhaps via coated pits, which are present on PMNs. Associated with receptor occupancy, phospholipases are activated and intracellular cAMP increases (not shown). Portrayed here is one of the pathways that appears to be activated by chemoattractants in leukocytes. Reproduced from Pike and Snyderman (1984).

absence of any other added phospholipids (McPhail et al, 1984). It is believed that the activation of phospholipases by chemo-attractants releases unsaturated fatty acids such as arachidonic acid, which can serve as second messengers to stimulate protein kinase C, which transduces certain chemotactic signals (Fig 2).

Movement Of The Cells

Chemo-attractant receptors on PMN can trigger a number of responses, as have been discussed, however the final outcome of these processes results in movement of the cell. The mechanism whereby the cell exhibits motility will be discussed fully, while dealing with ingestion.

PMN exposed to a gradient of a chemotactic factor become polarised, with the advancing front of the cell exhibiting a broad, thin veil (lamellipodium) while a knob-like tail and trailing retraction fibres are seen at the rear (Ramsey, 1972b, Zigmond and Hirsch, 1973). The nature of the contractile system producing pseudopods, cytoplasmic streaming and cell movement, has begun to be understood.

As neutrophils initiate movement, actin microfilaments increase in number and distribute themselves towards the leading portion of the moving cell (Oliver et al, 1978). For sustained movement to occur, the microfilament network must be continuously remodeled, actin polymerisation and microfilament production are important determinants of cell motility. Actin comprises

10% of neutrophil cytoplasmic protein (Boxer and Stossel, 1976) and has two interchangeable states with widely different polymerisation potentials (Harwell et al, 1980; Korn, 1982).

Much of what is known about the process of motility was discovered by studying macrophages. Stossel (1975, 1976, 1977) elucidated the organisation and co-ordination of the contractile elements of rabbit pulmonary macrophages. His findings are discussed in a later section.

Thus, the first step towards elimination of invading micro-organisms, chemotaxis, is extremely complicated itself composed of many stages, and although its various mechanisms begin to be understood, many questions about these processes remain to be answered.

1.2 Recognition.

Mammalian phagocytes have the capacity to discriminate about the material they phagocytose. They will engulf foreign cells, but reject homologous ones, they ingest some micro-organisms, but tend to overlook others, particularly encapsulated ones (Stossel, 1975).

The mechanism of recognition is poorly understood. Some knowledge has been gained about this process from the surface modifications brought about by the serum of higher organisms, opsonins.

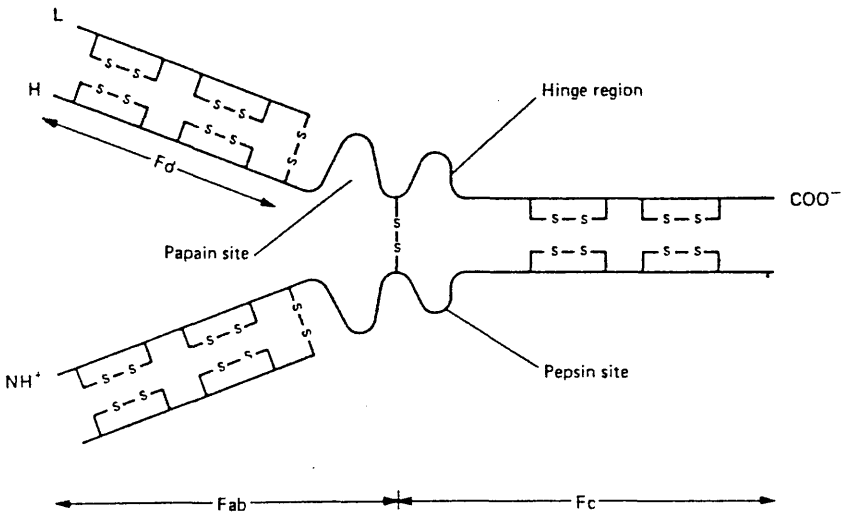


Figure 3. Diagrammatic presentation of the Ig molecule.

Opsonins

In 1903, Wright and Douglas observed that fresh serum promoted the ingestion of micro-organisms by phagocytes, they originated the term "opsonin", derived from the Greek "opsonein", meaning "to prepare food for". An opsonin has come to represent any agent in serum which acts on particles to increase their palatability to the phagocyte. From further studies, it was gathered that two forms of opsonins existed in serum, one heat-labile, the other heat-stable. The opsonic activity of normal human serum was found by Wright and Douglas (1903) to be reduced by exposure to temperatures above 50°C, with complete inactivation occuring in some cases when serum was heated to 60° - 65°C for 10-15 minutes. In contrast Neufeld and Rimpau (1904) demonstrated opsonins in immune serum which were effective in high dilution against the immunizing strain and were stable at 56°C.

It is now known that the heat-stable opsonins are immunoglobulins and the heat-labile opsonins are components of the complement system.

Immunoglobulin (Fig 3) acts both to identify the invading organism and to initiate adjacent complement activation.

These serum components are not mutually exclusive as opsonins, rather they function by acting through different mechanisms of opsonisation. Depending on the choice of test particle, the phagocytic cell type, and the opsonising medium, one

mechanism may be favoured over another.

Specific Antibody

Antibodies of either IgG or IgM class are able to act as opsonins (Robbins et al, 1965; Rowley and Turner, 1966; Smith et al, 1967) and IgA particularly in crevicular PMN (Fanger et al, 1983). It is not known whether they act by neutralising anti-phagocytic surface properties of bacteria or by forming a ligand between bacteria and phagocyte, or both. There is some evidence that they do not act solely by neutralising the anti-phagocytic surface properties of organisms (Rowley and Turner, 1966).

The antibody combines with the surface antigens of bacteria through the antibody combining site located in the $F(ab')_2$ portion of the molecule. (Fig 3) The F(c) portion of the molecule, which is critical to its function as an opsonin, (Quie et al, 1968) is then free to attach to specific receptors on the surface of the phagocyte (Messner and Selinek, 1970), and complete the bridge between bacteria and phagocyte.

The action of specific antibody alone is probably only significant in vivo in the hyperimmune animal with its large excess of specific antibody.

Complement

Complement is able to accelerate the rate of phagocytosis of

pneumococci opsonised by capsular antibody (Ward and Ender, 1933). It is thought that the sequence of events is parallel to that of the initial steps of the haemolysis of red blood cells by antibody and complement.

The bacterial antigen and opsonising antibody unite, the F(c) portion of the antibody then binds and activates the first component of complement (C1). C1 then activates the fourth (C4) and second (C2) components of complement. This bimolecular complex may activate and lead to the attachment of hundreds of molecules of the third component (C3). Receptor sites for activated C3 or C3b have been demonstrated on the surface of phagocytes (Huber et al, 1968; Lay and Nussenzweig, 1968). The C3b on the surface of bacteria acts as a ligand between bacteria and phagocytes.

The Alternative Complement Pathway (Properdin Pathway)

This appears to be a non-specific mechanism of opsonisation that is found in non-immune and germ-free animals, its activity is destroyed by heating the serum at 56°C for 30 minutes (Smith and Wood, 1969).

Some investigators have considered this system to represent the combined action of small amounts of "natural" antibody and complement (Slopex et al, 1962), others have suggested it is distinct from both antibody and complement (Hirsch and Strauss, 1964).

The pathway involves activation of C3 and fixation of C3b on bacterial cells surface (Shin et al, 1969). The activation of C3 occurs in the absence of C1, C4 and C2, therefore it does not follow the classical pathway of C3 activation, but rather follows an alternative pathway, the properdin pathway (Pillemer et al, 1954; Marcus et al, 1971).

The properdin pathway appears to be composed of properdin itself, a hydrazine sensitive factor (factor A) and a heat-labile factor (factor B-). The fifth component of complement (C5) has also been shown to participate in the pathway for pneumococci, but plays a minor role compared to C3 (Ruddy et al, 1972).

Thus although not completely understood, the properdin pathway represents a unique antibacterial defence system, which is composed of 7S immunoglobin and C3, and in which the activation of C3 proceeds via the alternate complement pathway.

1.3 Adhesion.

Adhesion depends on the surface properties of both particles and host cell. One of the factors which could play a role in cell adhesion is surface electrostatic charges. However, in most cases PMN and micro-organisms are both negatively charged and one would expect repulsive forces to prevent adhesion between them, as this does not happen, it may be concluded that electrostatic forces do not play the main role in adhesion or alternatively surface charge is modified by the presence

of charged ions (Weiss, 1969; Beveridge, 1980; Ryter and De Chastelier, 1983).

The specific and un-specific receptor sites that have been discovered in recent years play a more critical role. Un-specific binding refers to the adhesion of latex, oil-droplets and any undefined attachment, and appears to be mediated by the hydrophobicity of particles or molecules that can promote physical forces leading to adhesion between particle and cell surface (Stossel, 1975; Rabinovitch, 1967; Benoliel et al, 1980; Vogel et al, 1980).

In addition to hydrophobic forces, more specific binding sites have been found. Some of them behave as lectin receptors and recognise a specific sugar eg. glucose, mannose and are located on the phagocyte cell surface (Stahl et al, 1978; Vogel et al, 1980; Glass et al, 1981; Sung et al, 1983). Other binding sites seem to exist on pili. These proteinaceous thin filaments have been shown to favour adherence to various kinds of cells (Smith, 1977; Beveridge, 1980; Pearce and Buchanan, 1980).

Truly specific receptors were discovered on macrophages and leukocytes. Two receptors react specifically with the F(c) portion of IgG molecules (Silverstein et al, 1975; Unkeless et al, 1981). One F(c) receptor mediates the efficient binding and ingestion of IgG-antigen complexes (Arend and Mannik, 1972) and IgG-coated particles (Mantuani et al, 1972;

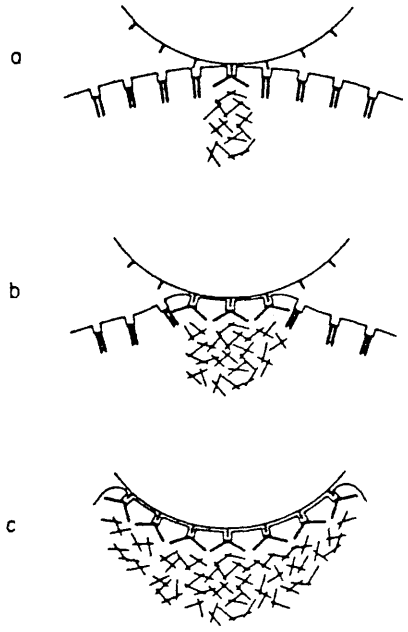


Figure 4. Model of proposed phagocytic mechanism (reproduced from Griffin et al (1975) | = IgG molecule or other ligand. Y = Fc receptor or other membrane receptor in an inactive state. Y = Fc receptor or other membrane receptor in an activated state. X = Cytoplasmic contractile proteins.

Griffin et al, 1975), the other F(c) receptor binds to sub-classes of IgG. (Fig 4). Immunoglobins of these sub-classes bind with high affinity to macrophages in the absence of antigens (Arend and Mannik, 1972). The other type of specific receptor found in "professional" phagocytes (PMN, mononuclear cells and tissue macrophages) binds to C3, one of the components of complement present in serum. C3 is an inactive precursor molecule consisting of a heavy and a light chain joined by disulphide bidges (Silverstein et al, 1977), specific proteases cleave a small fragment of the heavy chain, converting this inactive form into C3b, that specifically binds to C3 receptors (Reid and Porter, 1981). Both kinds of receptors are different and independent from non-specific receptors (Griffin and Silverstein, 1974; Michl et al, 1976). Some studies have shown that C3b receptors of both neutrophils and mononuclear phagocytes mediate attachment only, whereas F(c) receptors induce particle engulfment (Hed, 1981; Griffin, 1982; Hed and Stendahl, 1982). Others indicate that C3b also promotes ingestion (Muschel et al, 1977; Shaw and Griffin, 1981; Segerling et al, 1982). Griffin (1982) discussed the difficulties linked to these studies and possible reasons for contradictory results. He finally proposed as Ehlenberger and Nussenzweig (1977) that the "chief role of C3b and its receptors is to promote particle binding, the chief role of IgG and its receptors is to promote particle ingestion; the enhanced particle binding by C3b and its receptors serves to facilitate engagement of the cell's phagocytic signal-generating F(c) receptors by IgG."

Particle adhesion to cells bearing ligand surface receptors is generally temperature and energy-independent, whereas unspecific binding may not depend upon these factors (Griffin et al, 1975b; Benoliel, 1980; Glynn, 1981).

IgG-coated erythrocytes form tight and continuous contact areas with host cells surface (Kaplan, 1977; Benoliel et al, 1980), whereas C3 receptors establish discontinuous contacts (Munthe-Kaas et al, 1976; Kaplan, 1977). For unspecific or more specific binding sites, contacts differ according to the particle and cell. This shows that no general rules can be established on the mode of attachment to specific or unspecific receptors.

Although it is clear that certain well-defined serum proteins, IgG and activated C3 coat particles and render them ingestible, the physical and chemical properties of the coat have not been fully elucidated. It is known that antibody will bind to particles via the F(ab) region, and that papain- or pepsin-digested antibody will bind to particles, but lacks opsonic activity. It appears that the F(c) region of the immunoglobin activates ingestion and the C3b receptor appears to function primarily to bind the particle to the PMN.

Therefore F(c) and C3 receptors found on "professional" phagocytes' surfaces are of great importance in defence mechanisms. The many studies on the effects of opsonisation illustrate the importance of these receptors for microbe

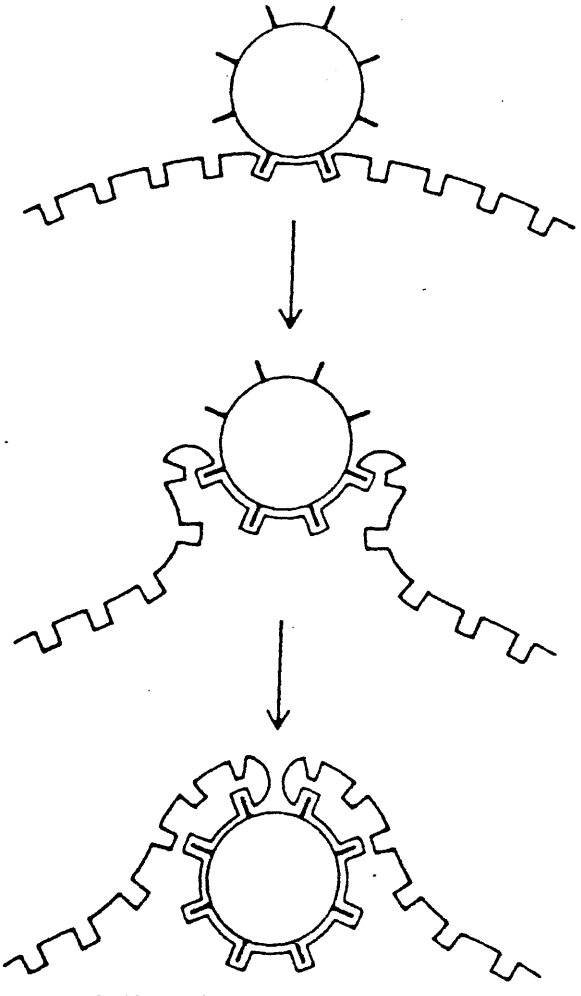


Figure 5. Diagram of the zipper mechanism of phagocytosis.
Top: Particle diffusely coated with serum ligands attaches to membrane receptors on the surface of a phagocyte.
Middle and Bottom: Additional receptor ligand interactions guide the movement of the phagocyte plasma membrane over the surface of the particle.
Reproduced from Silverstein et al (1977).

adherence and ingestion.

1.4 Ingestion.

Ingestion is the process by which particle(s) are captured by membrane extensions that surround and finally fully enclose the particles in phagocytic vacuoles.

Particle-associated immunoglobin and complement bind to receptor sites on the surface of the phagocyte, cellular pseudopods surround the particle by the process of circumferential adherence, and in this way the phagocyte membranes move about the particle and fully enclose it within a phagocytic vacuole. This has been termed the "zipper mechanism" of phagocytes (Griffin et al, 1976).

The zipper model (Fig 5) predicts that a particle with ligand distributed over its entire surface will bind continuously and circumferentially to receptors on the surface of the phagocyte and be totally engulfed, whereas particles with ligands on one portion of the particle surface will be bound, but not ingested by the phagocyte. Studies by Griffin (1976) confirmed this prediction. (Fig 6)

Ingestion is a temperature dependent and energy requiring process (Griffin, 1975; Stossel, 1975). Metabolic inhibitors or low temperatures permit binding of particles to the cell's surface in the absence of phagocytosis.

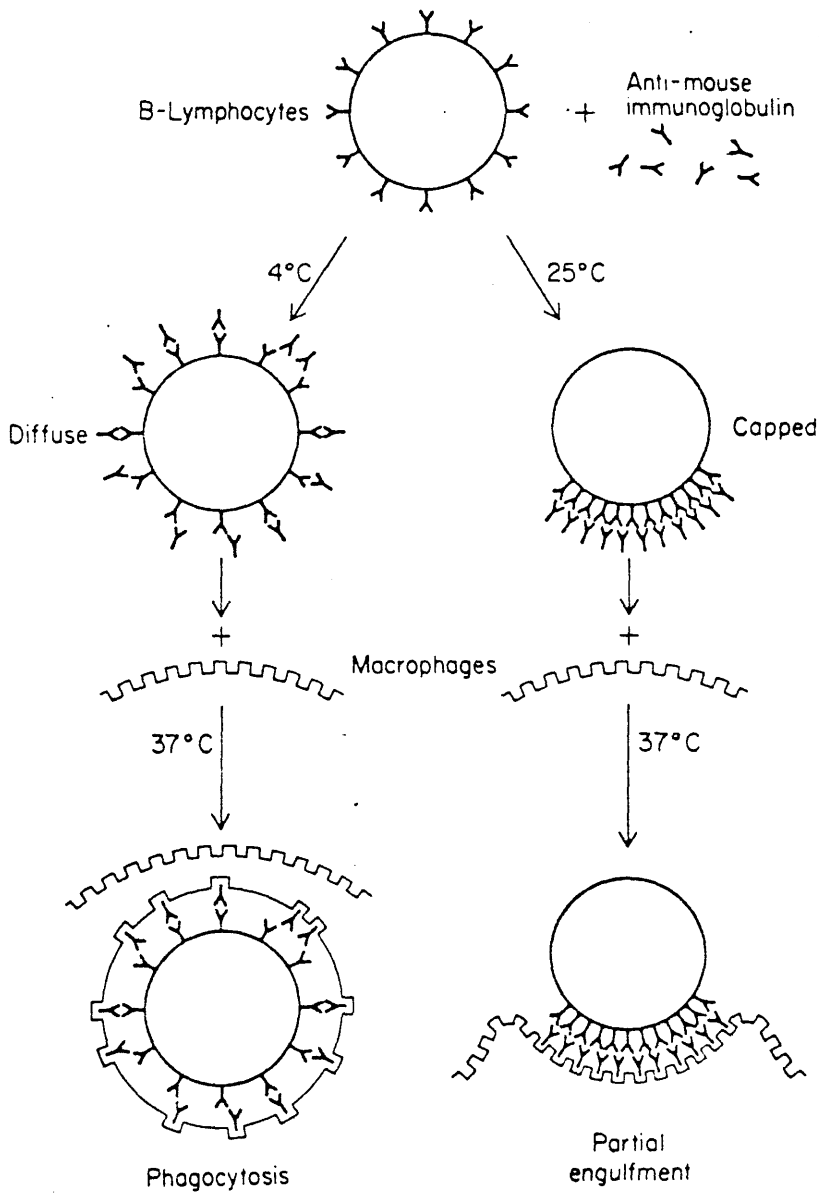


Figure 6. Evidence in support of the zipper model of phagocytosis. Particles with diffusely distributed ligands are ingested, whereas particles with capped ligands adhere to the surface of the phagocyte but are not ingested.
Reproduced from Silverstein et al (1977).

The Plasma Membrane

Since the initiating events of ingestion occur at the plasma membrane, various investigators have turned their attention to the PMN plasma membrane. Due to difficulties in achieving good yields of purified plasma membranes, more investigators turned to phagosomes, which are more easily isolated due to ingested material conferring unique densities to these structures (De Pierre et al, 1973).

Phagosomes, which include membranes of lysosomes that fuse with the phagocytic vesicle, have been purified from Acanthamoeba, fibroblasts, human, rabbit and guinea pig PMN and rabbit alveolar macrophages (Stossel, 1975). Comparative analyses of phospholipid composition of Acanthamoeba plasma membranes with phagosomal membranes of guinea pig and human PMN phagosomal membranes with total cellular membrane phospholipids failed to reveal striking differences in composition between the respective membranes (Stossel, 1975), suggesting that phagosomal membranes simply reflect the phospholipid content of the plasma membranes from which they are derived, despite the potential for membrane alteration by peroxidases and phospholipases available within the cell.

Surface charge could influence ingestion, once contact has occured, by its effect on interfacial tension (Ponder, 1928). However, Noseworthy and co-workers (1972) observed that treatment of intact guinea pig PMN with neuraminidase released

approximately 50% of the total sialic acid of the cell, (sialic acid being a contributor to the net negative surface charge of the PMN) without affecting either phagocytosis of glycolysis. This suggests that maintenance of the negative surface charge on neutrophils is not crucial to ingestion.

Phagosome Formation

Ligand attachment to membrane receptors on the phagocyte initiates changes in the submembrane region. Organelles are generally excluded from this area and a filamentous network forms. The process continues with sequential attachment of ligand to receptor until pseudopods fully enclose the particle in a vacuole consisting of internalised extracellular space surrounded by plasma membrane. The apposed membranes initially form a stalk which connects the vacuolar space with the outside of the cell; however fusion and lysis occur and the phagosome is set free. Klebanoff and Clark (1978) suggest that fusion and lysis may not be instantaneous; channels connecting the phagosome to the outside have been seen, with degranulation occuring both into the vacuolar space and into the stalk. The phagosome may thus remain connected to the surface of the cell by sheets of apposed plasma membrane for appreciable periods. Jacques and Bainton (1978) also noted this phenomenon whilst studying changes in pH in vacuoles. Cech and Lehrer (1984) specifically investigated this phenomenon and concluded that during phagocytosis of Candida albicans, 40% of the resulting vacuoles were unsealed.

In contrast, it is known that ingestion accelerates the rate at which phagocytes acylate lysophosphatides added to the medium bathing cells (Elsbach, 1972). Lysophosphatides can induce the fusion of cell membranes, and presumably are involved in phagolysosome formation.

Actin-myosin Contractile Filaments

On thin section electron micrographs, immediately below the membrane of the phagocytic cup, can be seen a thick zone from which all cytoplasmic organelles are excluded by a meshwork of filaments. This meshwork has been shown to contain actin, actin-binding proteins and myosin (Taylor and Condeelis, 1979). There is considerable evidence to suggest that these structures play an important role in the phagocytic process.

The organisation and co-ordination of the contractile elements of rabbit pulmonary macrophages have been extensively studied (Stossel and Hartwig, 1975, 1976, 1977). The contractile elements include actin- and myosin-containing microfilaments, actin-binding protein, and at least in macrophages, a cofactor protein that stimulates Mg^{2+} -dependent ATPase activity (Stossel, 1975) and gelsolin, a calcium-dependent regulatory protein (Yin *et al*, 1979).

Gelation occurs *in vitro* when actin filaments are cross-linked by actin-binding protein, a process controlled by gelsolin, so named because of its influence on reversible gel-sol transformations of actin filaments. A solid gel is formed at room

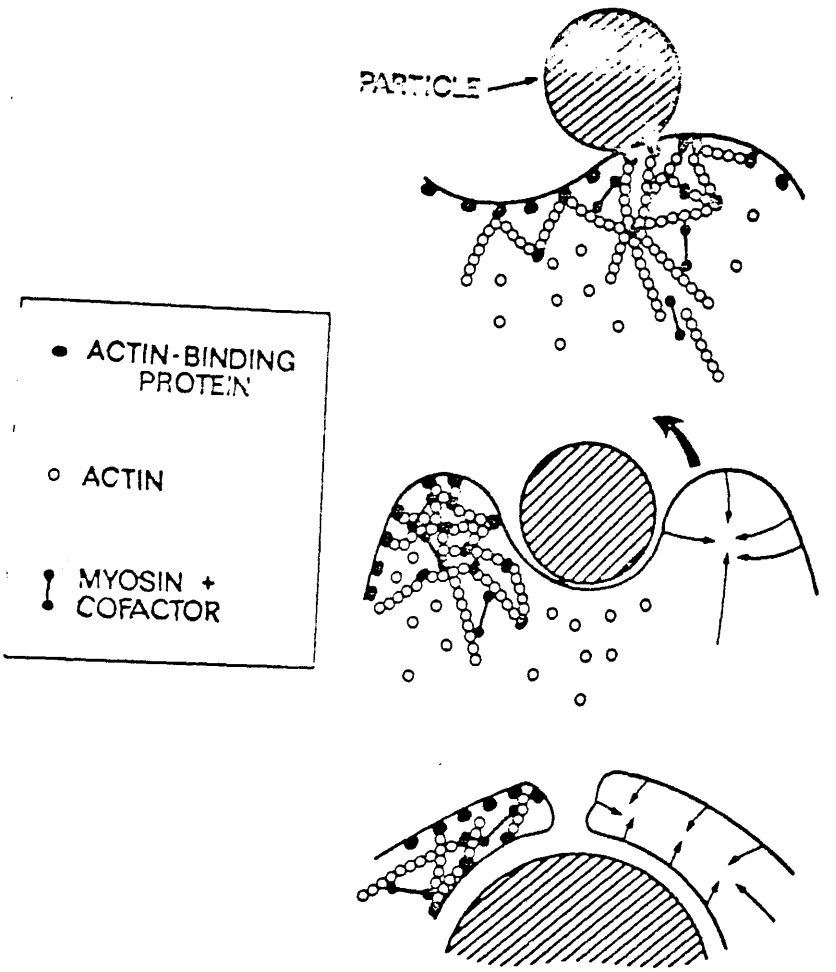


Figure 7. Proposed mechanism for pseudopod formation. Contact between particle and phagocyte causes the activation or release of actin-binding protein from the cell membrane, which initiates an interaction between actin and myosin plus cofactor. Gelation of the cytoplasm occurs and contraction of this cytoplasmic gel causes the formation of a narrow hyaline pseudopod. Reproduced from Stossel and Hartwig, 1976.

temperature, this process is inhibited by cytochalasin B, a compound which disrupts microfilaments and functions dependent on them. Cytochalasin B is known to inhibit phagocytosis by PMN (Klebanoff and Clark, 1978).

Contraction occurs *in vitro* when myosin contracts the actin gel into an aggregate, a process accelerated by the co-factor protein and occuring in the presence of Mg^{2+} and ATP.

Plasma membrane movement may occur as a result of gelation and contraction phenomena involving actin and myosin (Stossel, 1976). (Fig 7) Contact of the particle with the macrophage plasma membrane results in activation of the actin-binding protein. This protein causes the assembly and gelation of actin at the periphery of the cell and contraction of this gel to form pseudopodia is induced by myosin and the co-factor. Circumferential adherence of plasma membrane to the particle with the associated sol to gel transformation in the adjacent cytoplasm causes the pseudopodia to move gradually around the particle.

Although comparable studies have not been done in PMN the presence of actin and myosin in PMN and the description of a patient with recurrent bacterial infections, PMN actin dysfunction and depressed phagocytosis (Boxer *et al*, 1974) support a role for contractile proteins in the phagocytic process.

Microtubules do not appear to be as crucial to the phagocytic process in PMN as microfilaments. Colchicine, an inhibitor of mictotubule assembly, has been reported to inhibit PMN phagocytosis by some investigators, but not by others (Klebanoff and Clark, 1978).

It has been proposed that microtubules may serve in the control of membrane microviscosity (Berlin and Fera, 1977). Certain lectin-binding sites are selectively removed from the surface of PMN during phagocytosis, whereas other transport proteins are selectively excluded from the sections of invaginated membrane (Tsan and Berlin, 1971; Oliver et al, 1974). Colchicine at doses which do not affect the rate or extent of phagocytosis, prevent this movement of membrane proteins (Ukena and Berlin, 1972; Oliver et al, 1974). Phagocytosis by rabbit PMN was found by Berlin and Fera (1977) to decrease membrane microviscosity as measured by fluorescence depolarisation of lipophilic probes embedded in the membrane. This is a microtubule-dependent re-organisation of membrane lipids and proteins, leading to decreased microviscosity.

Role Of Calcium In Transmembrane Signalling

Griffin (1976) proposed that in the case of IgG-coated particles, ligand-receptor interaction generates a signal, perhaps the release of actin-binding protein from the plasma membrane, that would initiate polymerization and aggregation of contractile proteins resulting in the extension of pseudopodia. Pseudopod

extension would result in further receptor-ligand interaction, which in turn would elicit further contractile protein association. The factor(s) triggering the dissociation of the filament network once a particle has been ingested remain unknown. De Chastelier and Ryter (1981, 1982) showed in Dictyostelium discoideum that calcium deposits were especially abundant along the inner face of the filopod and phagocytic cup plasma membrane, that is where the membrane was underlayered with a filament network. They proposed that formation of calcium deposits was related to mobilisation of contractile proteins and not to their dissociation. These calcium deposits seem to result from a phosphatase activity, but their exact meaning is not yet established. The most attractive hypothesis is that they correspond to calcium channels, especially as many authors have shown that calcium promotes phagocytosis (Stossel, 1975; Hartwig et al, 1980) and is more abundant in actin filament-rich regions (Taylor et al, 1980).

In PMN, one of the first molecular events elicited by extracellular ligands is displacement of calcium from membraneous stores into the cytosol (Naccache et al, 1979; Mottola et al, 1982) where it can be extruded by a high-affinity ATP-dependent pump localised in the plasma membrane (Mottola et al, 1980, 1982).

Histochemical and kinetic evidence suggests that the site of calcium mobilisation is the area on the plasma membrane involved in interaction with the stimulant (Hoffstein, 1979; Mottola, 1980, 1982). Mottola (1980), using phorbol myristic acetate

(PMA) showed mobilisation of membrane Ca^{2+} can cause an increase in cytosolic free ion concentration from 10^{-7} - 10^{-8}M to 5 X 10^{-5}M within a few seconds. This enhancement in cytosolic calcium is sufficient to activate several calcium-dependent processes, including the calmodulin-dependent superoxide-forming enzyme, protein kinase(s) and gel-sol transformation of actin.

Stossel et al (1975) purified a calcium-binding protein called gelsolin from macrophages. This protein severs actin filaments and dissolves the gels of cross-linked actin at Ca^{2+} concentrations exceeding 2 X 10^{-7}M.

Romeo (1982) proposed that a sequence of Ca^{2+} mobilisation from the plasma membrane, diffusion into nearby cytoplasm and extrusion from the cell by the plasma membrane Ca^{2+} pump could create gradients of Ca^{2+} concentrations in the cortical cytoplasm. Thus if gelsolin was also present in neutrophils, a discontinuity in peripheral distribution of Ca^{2+} could cause a discontinuity in the gel-sol state of actin, with water moving from the less to more cross-linked domains.

Hence fluctuations in Ca^{2+} concentration in the cell cortex could control pseudopod development and ingestion of particles.

Energy Requirements

The ingestion process is dependent on metabolic energy. It

is generally believed that the source of this energy in PMN is ATP generated by glycolysis (Jemelin and Frei, 1970).

Studies with various inhibitors of glycolysis such as iodoacetate, fluoride and deoxyglucose, reviewed by Klebanoff and Clark (1978) suggests that glycolysis is required for optimum phagocytosis. Active glycolytic activity may not be required for phagocytosis if adequate pre-formed ATP is present (Bodel and Malawista, 1969). Phagocytosis by PMN is associated with heat (Levin, 1973) and chemiluminescence (Allen et al, 1972) production, and the rapid depletion of cellular ATP (Bodel and Malawista, 1969). Few mitochondria are present in the mature PMN, and hypoxia or inhibitors of oxidative phosphorylation such as dinitrophenol do not inhibit the ingestion process (Becker et al, 1958; Sbarra and Karnovsky, 1959; Cohn and Morse, 1960).

1.5 Degranulation.

A critical aspect of the phagocytic process is the concentration within the phagocytic vacuole of agents which are toxic to the ingested micro-organism. These agents include granule components as well as metabolic products. During particle ingestion these constituents are released into the extracellular fluid. The process of degranulation involves the delivery of granule components into the phagosome or the intracellular space. After particle ingestion, granules adjacent to the phagocytic vacuole fuse with the vacuole, the common membrane ruptures and the granule contents are

discharged into the vacuolar space, converting the phagosome into a phagolysosome. Both specific granules and azurophilic granules participate in the degranulation process. If the phagocytic load is large, there can be almost complete disappearance of granules from the cytoplasm (Klebanoff and Clark, 1978).

Initiation Of The Respiratory Burst

The respiratory burst refers to a co-ordinated series of metabolic events that take place on stimulation of the PMN plasma membrane by a variety of stimuli including bacteria, surface active agents (Graham et al, 1967; Kakinuma, 1974), such as fatty acids and digitonin (Graham et al, 1967; Kakinuma, 1974), lectins such as concanavalin A (Romeo et al, 1973) and phorbol myristate acetate (PMA) (Repine et al, 1974). These events are responsible for all oxygen dependent killing by phagocytes, and include increased oxygen consumption by the cell (Baldridge and Gerald, 1933; Sbarra and Karnovsky, 1959), increased oxidation of glucose via the hexose monophosphate shunt pathway (HMPS) (Sbarra and Karnovsky, 1959), increased production of hydrogen peroxide (H_2O_2) (Iyer et al, 1961; Root et al, 1975), production of superoxide anion (O_2^-), (Babior et al, 1973; Curnette and Babior, 1974) and generation of chemiluminescence (Allen et al, 1972).

The initiation of the respiratory burst does not require either phagocytosis or degranulation, but simply contact between the phagocyte and stimulant (Curnette et al, 1979).

A number of different types of membrane perturbations will activate the respiratory burst independent of phagocytosis. These include PMA, detergents, concanavalin A, complement components (Goetzl and Austen, 1974) and non-ingestible particles such as mycoplasma (Simberkoff and Elsbach, 1971). Therefore phagocytosis is not a necessary pre-requisite for the oxidative responses.

After contact the conversion time from the resting state to the fully stimulated state is approximately 20-70 seconds (Cohen and Chovaniec, 1978; Segal and Coade, 1978; Newburger et al, 1980). This event corresponds to the induction of a plasma membrane enzyme, the NADH or NADPH oxidase (Ryter and De Chastelier, 1983).

Romeo et al (1975) hypothesised that the trigger mechanism for stimulating the respiratory burst consists of a rapid shift of divalent cations from the environment and/or plasma membrane binding sites towards "special zones of the cytoplasm". This model is supported by the known requirement for extracellular Ca^{2+} or Mg^{2+} for O_2^- production to occur (Schell-Frederick, 1974; Romeo et al, 1975; Curnette et al, 1979; Lehmeyer, 1979). Various ionophores are able to stimulate the oxidative metabolism of these cells in the presence of exogenous Ca^{2+} or Mg^{2+} (Schell-Frederick, 1974; Romeo et al, 1975). Phosphatidylinositol breakdown has been implicated in the control of the Ca^{2+} channels in a wide variety of tissues (Michell, 1975; Michell et al, 1976). This early

event in Ca^{2+} transport is commonly linked to an increased labelling of phosphatidylinositol, due to its subsequent resynthesis. An increased labelling of this phospholipid, plus phosphatidic acid, during phagocytosis by guinea pig neutrophils has been reported (Karnovsky and Wallach, 1961; Sastry and Hokin, 1966).

The precise mechanism of the initiation of the respiratory burst therefore remains to be clarified, but transmembrane signalling mechanisms are proving to be extremely complex.

1.6 The Microbicidal Oxidase System.

The Primary Oxidase

The identity of the enzyme responsible for the primary oxygen-consuming reaction of the respiratory burst has remained controversial for many years. It is agreed that the enzyme catalyses the reduction of oxygen to superoxide by a reduced pyridine nucleotide. There is conflicting evidence as to whether the immediate electron donor is NADH or NADPH. However, it is believed by most authors that the evidence favours NADPH (Babior, 1978; Badwey and Karnovsky, 1980; Michell, 1983; Segal, 1984). The activity of the HMPS, the main pathway of electrons to NADPH, is markedly increased in association with oxidase activity (Zatti and Rossi, 1965). In addition, impaired function of this pathway due to extreme deficiency of the key enzyme glucose-6-phosphate dehydrogenase results in failure of oxidase activity (Gray et al, 1973).

The terms NADH and NADPH oxidase have been superceded by the increased understanding of the mechanism responsible for the reduction of oxygen. It is accomplished by an electron transport chain terminating in an unusual b type cytochrome (Segal and Peters, 1976; Segal and Jones, 1978, 1979a,b, 1980; Light et al, 1981; Segal et al, 1981; Borregaard et al, 1982, 1983; Jones, 1982; Michell, 1983; Borregaard and Tauber, 1984; Segal, 1984).

Extensive studies have shown that it has the lowest midpoint potential of any mammalian cytochrome b ($E_{m,7.0}$=-245 mV) (Cross et al, 1981), it binds carbon monoxide and has the oxidation-reduction characteristics of a true oxidase (Cross et al, 1981, 1982), it is found in neutrophils, monocytes, macrophages and eosinophils but none of the "non-professionally" phagocytic cells of man and a variety of other mammals and fish (Segal et al, 1981).

The cytochrome is located in the plasma membrane of neutrophils (Segal and Jones, 1979). Neutrophils also have a second pool of the cytochrome in the membrane of the specific granules which fuse with the plasma membrane, probably that lining the phagocytic vacuole, in association with degranulation (Segal and Jones, 1970; Borregaard et al, 1983; Garcia and Segal, 1984). There is disagreement as to whether the complement of b cytochrome in the plasma membrane of the neutrophil occurs de novo, or only as a result of specific granule fusion. Cytochrome b_{-245} becomes reduced when the

cells are stimulated with PMA in the absence of oxygen and is rapidly re-oxidised when oxygen is re-introduced into the system (Segal and Jones, 1960, 1979). It has been purified to homogeneity and has a molecular weight of 70,000 (Harper et al, 1984).

Abnormalities of this cytochrome have been clearly implicated in the pathogenesis of Chronic Granulomatous Disease, a syndrome in which the patients are unusually susceptible to infection (Segal et al, 1983).

A FAD-containing flavoprotein appears to be another component of this electron transport chain. It probably donates electrons to the cytochrome b. It has a similar subcellular distribution to the cytochrome and largely co-purifies with it. However, it is a separate molecule as it is known to demonstrate a different pattern of detergent extraction from membranes (Segal, 1984).

It has also been suggested that ubiquinone is an electron carrier in this system (Sloan et al, 1981; Cunningham et al, 1982; Crawford and Schneider, 1983, 1982), however Segal et al, (1984) believed that most ubiquinone in leukocyte preparations comes from contaminating mononuclear cells and platelets, and that in PMN the only detectable quinone is located in mitochondria.

Michell (1983) proposed a hypothetical pathway for the electron

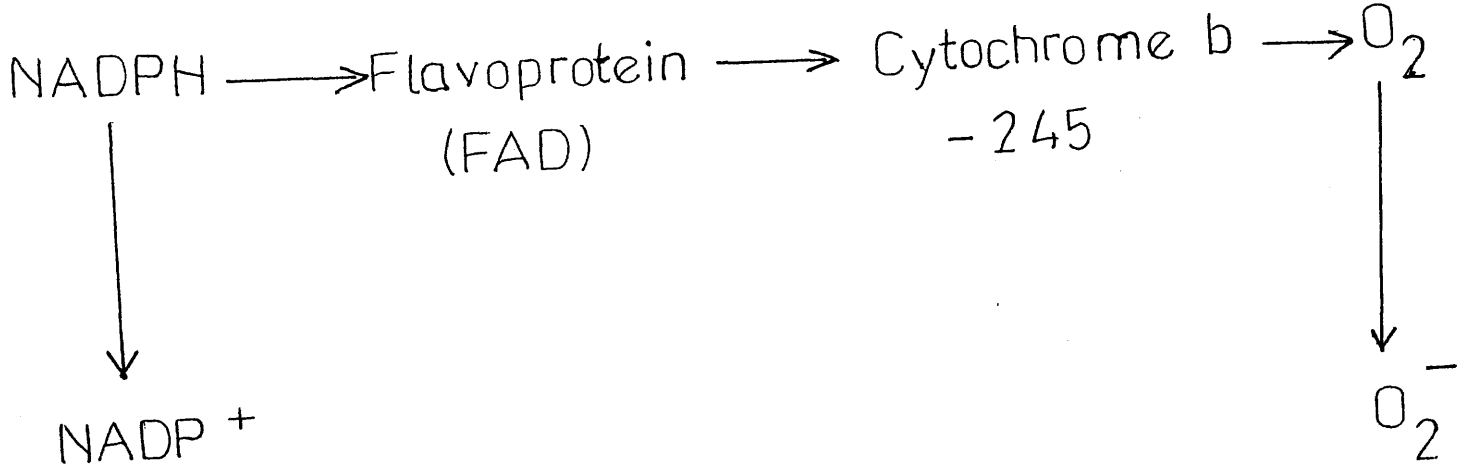


Fig 8 Hypothetical pathway for the electron transport chain in PMN. (Michell, 1983).

transport chain. (Fig 8)

Active Oxygen Species

During phagocytosis, PMN produce substantial quantities of superoxide and hydrogen peroxide. These substances may subsequently interact to produce hydroxyl radicals and singlet oxygen. Their formation and role in the bactericidal function of PMN has been extensively investigated.

Hydrogen Peroxide

Hydrogen peroxide is formed by the reaction of O_2^- with itself, either spontaneously, or under catalysis by superoxide dismutase: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

H_2O_2 has a certain amount of bactericidal potency, and has been proposed to act in conjunction with ascorbic acid (Miller, 1969) and certain metal ions to kill ingested micro-organisms by non-enzymatic means (Repine et al, 1981). The toxicity of H_2O_2 is increased in the presence of certain low molecular weight reducing substances, such as iodide (Klebanoff and White, 1969).

Karnovsky et al (1981) determined that H_2O_2 could be detected on the cell surface and within the phagosome, following phagocytic stimulation of the respiratory burst. Thus internalisation of the plasma membrane, with components capable of generating H_2O_2, accounts for the presence of H_2O_2 within the phagosomes.

H_2O_2 And Myeloperoxidase

Myeloperoxidase (MPO), a haemprotein with molecular weight of approximately 150,000, is an enzyme present in high concentrations in PMN. It catalyses the oxidation of halide ions to hypohalite ions by H_2O_2: $X^- + H_2O_2 \longrightarrow XO^- + H_2O$

Cl^-, Br^- and I^- can all be oxidised by this enzyme (Klebanoff, 1967; Harrison et al, 1976).

Klebanoff (1967) showed that H_2O_2, myeloperoxidase and a halide formed a potent anti-microbial system, the peroxidase greatly augmented the killing power of H_2O_2.

It is likely that Cl^- is the most important halide involved in the myeloperoxidase system in PMN, as it is the most abundant physiologically within the cell. Incubation of bacteria with H_2O_2, myeloperoxidase and Cl^- results in efficient killing at H_2O_2 concentrations as low as 10μM. In the absence of myeloperoxidase, similar levels of killing are not achieved until the concentration of H_2O_2 reaches 0.5mM, (Babior, 1978).

Several mechanisms have been proposed to explain bacterial killing by the H_2O_2-halide-MPO system. Early research using I^- as the halide ion, showed incorporation of iodine into the bacterial cell wall (Klebanoff, 1967). Similar experiments with radio-active Cl^- have shown that chlorine can be incorporated

into bacteria both by whole neutrophils and by the H_2O_2-halide-MPO system (Zgliczynski and Stelmaszynska, 1975). It was postulated that loss of integrity of the halogenated cell wall brought about cell death, however the correlation of halogenation and bacterial death is indefinite (McCall et al, 1971). Therefore although halogenation could contribute to cell death under some circumstances, this is not the only mechanism by which the MPO system kills bacteria.

The myeloperoxidase system is capable of decarboxylating amino acids, converting them to aldehydes, carbon dioxide and ammonia $R\text{-}CH(NH_2)\text{-}COOH \rightarrow R\text{-}CHO + CO_2 + NH_3$. Zgliczynski et al (1968) postulated that organisms were killed by the toxic aldehydes produced by this reaction. However, it has since been found that free aldehydes are not toxic enough to account for the observed bacterial killing (Paul et al, 1970). Another version of this mechanism postulates that amino acid constituents of the bacterial cell wall are degraded by this reaction, with disruption of the bacterial surface and cell death (Strauss et al, 1971). However, bacteria are killed efficiently by the H_2O_2-halide-MPO system even under conditions in which amino acid decarboxylation is completely inhibited (McCall et al, 1971), indicating that amino acid decarboxylation, if at all relevant, only plays a minor role in killing.

It is known that the H_2O_2-MPO-Cl^- system is able to generate a number of other toxic oxidant species. It is thought that hypochlorous acid (HOCl) is the most important of these

(Weiss et al, 1982).

$$H_2O_2 + Cl^- \xrightarrow[H^+]{\text{myeloperoxidase}} HOCl + H_2O$$

HOCl is a powerful oxidant capable of reacting with a wide variety of biological substrates (Selvaraj et al, 1980; Albrich et al, 1981; Weiss and LoBuglio, 1982). Albrich and Hurst (1982) reported that as little as 1nM of HOCl could kill 4×10^6 E.coli.

"Activated chloride" (Cl^+) species generated as above can react with nitrogenous compounds to form chloramines (Zgliczynski et al, 1971, Lampert and Weiss, 1983). Klebanoff and Clark (1978) postulated a number of ways in which chloramine formation could contribute to the death of micro-organisms. They suggested that chloramines may be directly toxic to the micro-organism, that chloramines hydrolyse continuously with the release of activated chloride (Sykes, 1965), thus acting as storage forms of available chlorine, prolonging the toxicity of the peroxidase system, and that degradation of chloramines may result in the conversion of unstable compounds to substance(s) with anti-microbial properties, such as aldehydes, as has already been discussed.

The precise mechanism(s) involved in bacterial killing by the H_2O_2-MPO-Cl^- system remains unclear, but it seems likely that a number of processes are involved. Current ideas seem to favour hypochlorite and other oxidised derivatives of chloride

as the anti-microbial agents.

Superoxide Anion

The superoxide anion is generated as a result of the reduction of molecular oxygen by the electron transport chain in PMN. The O_2^- is localised on the plasma membrane and as such is present in phagosomes, after phagocytosis has taken place. Thus it was believed that the superoxide anion played a role in the destruction of invading micro-organisms (Babior et al, 1973). This idea has since been disputed. Evidence from experiments using artificial O_2^--generating systems has shown that, except under unusual circumstances, O_2^- plays a very minor role in bacterial killing (Gregory et al, 1973; Gregory and Fridovich, 1974). The local rates of O_2^- produced by, for example the xanthine-xanthine oxidase O_2^--generating system is orders of magnitude smaller than the rates characteristic of stimulated PMN. However, Roos (1984) has shown that "cytoplast" preparations of PMN which are greatly depleted of granule proteins, but which still phagocytose bacteria and produce a respiratory burst, fail to kill S. aureus and E. coli normally. This is a strong indication that O_2^- and H_2O_2 have a limited toxic effect in the absence of granule proteins. Additional support for this is the evidence from Klebanoff (1974), using the xanthine-xanthine oxidase system, he found that E. coli, S. aureus and Candida tropicalis grown in complete medium are unaffected by O_2^-, however when this system was supplemented with MPO and additional chloride,

the organisms were rapidly killed. Thus O_2^- alone does not contribute significantly to the microbicidal capacity of the PMN.

Singlet Oxygen

Singlet oxygen is another highly reactive species which has been implicated in the killing of bacteria by PMN.

Upon absorption of energy, the outer electrons of oxygen can change their spin and occupy the same or different orbitals. Thus excited states of oxygen can be formed, termed singlet oxygen ($^1O_2$). Although it has the same molecular formula as atmospheric oxygen (O_2), it differs from the latter in the distribution of the electrons around the two oxygen nuclei (Kearns, 1971). In atmospheric oxygen, the electrons form a cylindrical cloud whose axis is the line joining the nuclei. In singlet oxygen this cloud is distorted away from a cylindrical configuration. This distortion so alters the chemical properties of the molecule that it confers entirely different patterns of reactivity. Singlet oxygen is far more reactive than molecular oxygen. It attacks especially molecules that contain carbon-carbon double bonds. The reactive nature of singlet oxygen means that it is capable of inflicting lethal damage on biological systems.

A number of reactions are capable of generating singlet oxygen under conditions that may be physiologically relevant.

Klebanoff (1980) reviewed these reactions:-

(A) $HO_2\cdot + O_2^- + H^+ \longrightarrow {}^1O_2 + H_2O_2$

(B) $O_2^- + H_2O_2 \xrightarrow{\text{metal}} {}^1O_2 + OH^- + OH\cdot$

(C) $OCl^- + H_2O_2 \longrightarrow {}^1O_2 + Cl^- + H_2O$

(D) $O_2^- + OH\cdot \longrightarrow {}^1O_2 + OH^-$

(E) $2O_2^- + R\text{-}\overset{O}{\overset{\|}{C}}\text{-}O\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}R \longrightarrow 2\,{}^1O_2 + 2RCO_2^-$

The reactions include the dismutation of O_2^- (A). Production of $^1O_2$ by this reaction is controversial (Kahn, 1970, 1976). The modified Haber-Weiss reaction (B) is believed to be responsible for the production of both OH. and $^1O_2$. The interaction of hypochlorite (OCl^-) with H_2O_2 (C) is of particular interest as hypochlorous acid is produced by the MPO-H_2O_2-halide antimicrobial system (Harrison and Schultz, 1976). $^1O_2$ may also be formed from the interaction of O_2^- with either hydroxyl radicals (D) or diacyl peroxides (E) (Danen and Arudi, 1978).

Evidence for the role of singlet oxygen in bacterial killing has been difficult to gather due to unsatisfactory methods for its detection. However Krinsky (1974) reported that human granulocytes are incapable of killing wild-type Sarcina lutea in vitro over a 90 minute period in the presence of serum. A mutant strain that lacks carotenoid pigments was rapidly killed under similar circumstances. Since carotenoids function as efficient quenchers of $^1O_2$, these data are suggestive both of the production of $^1O_2$ by

these cells and of a role for the latter as a bactericidal agent. A direct chemical determination of $^1O_2$ production by phagocytosing leukocytes has not yet been performed for any cell type.

Hydroxyl Radicals

The hydroxyl radical (OH.) is a highly unstable oxidising species that reacts almost instantaneously with most organic molecules that it meets.

The first evidence that human neutrophils produce OH. during phagocytosis came from studies in which the bactericidal activity of granulocytes was inhibited by superoxide dismutase (SOD), catalase and scavengers of OH. (mannitol and benzoate) (Johnston et al, 1975). This action of either catalase or SOD is consistent with an interaction between H_2O_2 and O_2^- to generate OH., which also produces $^1O_2$ (See reaction (B) in previous section). Chemical evidence was also reported by Tauber and Babior and independently by Weiss and co-workers in 1977. However, their results were based on the assumption that conversion of methional to ethylene indicates that there has been OH. formation, but unless they could show participation of the Haber-Weiss reaction, it did not constitute proof that OH. had been produced. Weiss et al, 1978 re-examined this question and using a system that employed an alternative substrate, the non-aldehyde 2-keto-4-thiomethylbutyric acid, showed ethylene production and that it was substantially inhibited by both catalase and SOD.

A possible reason for the discrepancy between the two methods is that methional, like all aldehydes, will oxidise spontaneously to a hydroperoxide, a class of compound that reacts rapidly with O_2^- to produce OH. (Babior, 1978). With 2-keto-4-methylthiobutyric acid, spontaneous oxidation to a hydroperoxide is not possible, so that H_2O_2 becomes a necessary participant in OH. formation. These findings suggest that OH. may be formed in phagocytes by a reaction between O_2^- and a peroxy compound: $O_2^- + R\text{-}OOH \rightarrow OH. + OR- + O_2$

Repine et al (1981) implicated the hydroxyl radical in the killing of S.aureus. The OH. being formed from H_2O_2 reacting with staphylococcal iron by the Fenton reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH. + OH-$

They found that growing the organism in increasing concentrations of iron increased their content of iron and dramatically increased their susceptibility to killing by H_2O_2. In addition to this they used a number of OH. scavengers and found that they inhibited H_2O_2-mediated killing. The extent of the inhibition was in direct relation to their effectiveness as OH. scavengers.

There is evidence from spin trapping studies to suggest that OH. radicals are generated within the vacuole (Green et al, 1979). This might be stimulated or inhibited by lactoferrin, depending upon the degree to which it is saturated with iron (Ambruso and Johnston, 1981; Gutteridge et al, 1981).

OH. and $^1O_2$ have been implicated as causitive agents in lipid peroxidation (Kellog and Fridovich, 1975; Lynch and Fridovich, 1978). They are also known to cause oxidation and damage to nucleic acids (Myers, 1973; Ito, 1978).

Therefore it is probable that OH. and $^1O_2$ play a role in bacterial killing, but the extent of this role is as yet unknown.

1.7 Oxygen-independent Anti-microbial Systems.

The azurophilic and specific granules are known to contain a number of enzymes and proteins which play a role in bacterial killing in the phagosomes. These include lysozyme, lactoferrin, acid and alkaline phosphatase, a number of hydrolases, and many granule cationic proteins (Klębanoff and Clark, 1978).

The role of lysozyme, lactoferrin and alkaline phosphatase will be considered in some detail.

Lysozyme

In 1922, Alexander Fleming discovered a substance in his own nasal mucus capable of dissolving or lysing certain bacteria. The substance, which turned out to be an enzyme was named "lysozyme" and was found to be widely distributed in nature (Fleming, 1922). Of all the materials examined, egg white was the richest source of lysozyme (Fleming, 1922).

Fleming also isolated a gram positive coccus which he called Micrococcus lysodeikticus ("lysis indicator") and this organism proved to be particularly susceptible to the lytic action of lysozyme.

The enzyme could only be defined by its ability to lyse certain bacteria until Meyer et al (1936) discovered that lysozyme digestion of bacterial substrates was accompanied by a liberation of reducing groups. Hallauer et al (1929) reported that lysozyme action was accompanied by a liberation of amino nitrogen. Various groups then went on to find lysozyme activity in egg white, saliva, tears, PMN, ficus and papaya extracts (Meyer, 1936; Barnes, 1940; Epstein and Chain, 1940).

In 1963, Jollès and Canfield independently elucidated the primary structure of the low molecular weight (14,500) enzyme which is comprised of a single chain of 129 amino acids (Jollès, 1960; Jollès et al, 1963), and in 1965 David Philips and his colleagues described the three-dimensional structure of lysozyme, based on X-ray crystallographic studies.

In 1952, Salton showed that the substrate for lysozyme in M. lysodeikticus is the cell wall mucopolysaccharide, a complex material containing both sugars and peptides, known as peptidoglycan. Subsequently similar glycopeptides were found in all other bacteria examined. The bacterial cell wall basically consists of linear polysaccaride chains containing

repeating units of N-acetylglucosamine and N-acetylmuramic acid residues in β 1-4 linkage (Strominger and Ghuysen, 1967). A variable number of acetylmuramic acid residues have tetrapeptide chains attached in N-acetylmuramyl-L-alanine linkage. Cross links between tetrapeptide chains result in the formation of a 3-dimensional net, the tightness of which will depend on the proportion of the N-acetylmuramic acid residues which contain tetrapeptide chains and on the extent of the cross-linkage. Lysozyme hydrolyses the glycosidic bond between acetylmuramic acid and acetylglucosamine. It must penetrate the mesh and hydrolyse these bonds in sufficient numbers to solubilise the cell wall peptidoglycan. Organisms vary widely in their susceptibility to the lytic action of lysozyme based on differences in the structure of the cell wall material.

Lysozyme is present in both the specific and azurophilic granules of PMN. It is also present in mononuclear cells (Bretz and Baggiolini, 1974; Spitznagel et al, 1974). Presumably it is released into the phagocytic vacuole following phagocytosis. Although a wide variety of bacteria are sensitive to the action of lysozyme, resistance is frequently encountered. For example, group A streptococci, staphylococci and almost all gram negative organisms resist the action of lysozyme unless treated concomitantly in some other manner (Root and Cohen, 1981). To be active, lysozyme must reach the glycosidic bonds and cleave enough of them to bring about fracture of the murein backbone of the peptidoglycan (Strominger and Ghuysen, 1967). The presence or absence of a protective outer

envelope, the extent of cross-linking and length of cross-bridges, as well as the O-acetylation of the peptidoglycans, are all factors that can modify lysozyme activity (Repaske, 1956; Brumfitt, 1959; Freese et al, 1973). Other physical procedures or chemical agents that increase the susceptibility of organisms to lysozyme include, the serum antibody-complement system, a combination of H_2O_2 and ascorbate (free radical generation), and both high and low pH. Treatment of bacteria with EDTA, a variety of antibiotics active against cell walls, proteases, butanol extraction and Triton X (a non-ionic detergent) render these organisms more susceptible to the action of lysozyme (Klebanoff and Clark, 1978; Root and Cohen, 1981). It is believed that such treatments affect the cell envelope of gram negative species or alter the cell wall itself, allowing lysozyme access to its substrate.

While most bacteria of clinical significance are resistant to lysozyme itself, the major function of this enzyme may be to act in conjunction with other neutrophil factors. It is thought that the primary function of lysozyme may not be as a bactericidal agent, but rather its most important function is the degradation of already phagocytosed bacteria.

Lactoferrin

Lactoferrin is a high molecular weight protein (77,000), long known to be present in milk (Sorenson and Sorenson, 1939; Schafer, 1951), and a variety of other biological fluids (Masson et al, 1966). It has also been recovered from human,

guinea pig, and rabbit PMN, and appears to be packaged in the specific granules (Baggiolini et al, 1970; Green et al, 1971; Pryzwansky et al, 1978).

The identification of lactoferrin in biological fluids was made possible through the use of a rabbit antiserum specifically reacting with this protein. Masson and Heremans (1966) detected lactoferrin in parotid saliva, bronchial secretions, nasal secretions, tears, seminal fluid and mucus from the uterine cervix.

Lactoferrin is an iron-binding protein. It retards the growth of bacteria and fungi in vitro (Masson et al, 1966; Oram and Reiter, 1968; Kirkpatrick et al, 1971; Bullen et al, 1972). It has been suggested as a possible contributor to defense against local mucosal infections.

The antimicrobial action of lactoferrin has been attributed to its ability to chelate iron, an action that makes this essential nutrient inaccessible to invading micro-organisms (Weinberg, 1974; Arnold et al, 1977). When not fully iron-saturated, bovine, human and goat lactoferrins were bacteriostatic for Bacillus stearothermophilus and Bacillus subtilus in both the presence and absence of trace amounts of metal. The bacterio·static action of lactoferrin was suppressed by Fe^{2+} and enhanced by Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+} (Oram and Reiter, 1968). Masson and Heremans (1966) found iron-poor preparations of lactoferrin displayed bacteriostatic activity against a variety of microbial species, including

S. albus, S. aureus, and Ps. aeruginosa. This effect was abolished by the addition of ionised iron.

The ready reversibility of this antimicrobial effect when an excess of iron is supplied to the deprived organisms suggests a simple bacteriostatic effect for lactoferrin. However, Arnold et al (1977), using purified iron-free (APO) human lactoferrin found the growth of Streptococcus mutans totally inhibited by prior incubation with lactoferrin, Vibrio cholera was similarly inhibited, but E. coli was not inhibited by apo-lactoferrin concentrations as high as 80μM. Saturated lactoferrin in similar concentrations did not affect growth of any of these bacteria. Thus it was suggested that lactoferrin is capable of exerting a direct bactericidal effect based upon its chelating properties. Various organisms have been shown to be highly susceptible to the bactericidal action of lactoferrin at concentrations within the physiological range of many secretions.

However, studies by Ambruso and Johnston (1981), suggested another way in which lactoferrin may contribute to the antimicrobial function of PMN. They found that iron-saturated human milk lactoferrin and human neutrophil lactoferrin enhanced ethylene generation from α-keto-γ- methiolbutyric acid. This was taken as a measure of OH. production. They found that iron bound to lactoferrin was about 5,000 times more effective in producing an enhancement in ethylene production than iron derived from $FeCl_3$ or ferric EDTA.

(The OH. is believed to be produced by an interaction between O_2^- and H_2O_2 in the presence of iron). The ability of neutrophil lactoferrin to provide iron efficiently to the oxygen radical-generating systems is compatible with a role for lactoferrin as regulator of OH. production.

Plasma lactoferrin levels are known to increase during inflammation, this is accompanied by a decrease in the PMN lactoferrin content (Hansen et al, 1975; Wright and Gallin, 1979). Bacterial sepsis and burn injuries are associated with loss of specific granules from PMN and defects in PMN chemotaxis (Wright and Gallin, 1979). Since lactoferrin can bind to other blood cells, it seems likely that this same highly charged molecule could participate in the PMN hyper-adherent response upon chemotactic stimulation (Van Snick and Masson, 1976; Broxmeyer et al, 1978). Oseas et al (1981) showed that the PMN lysosomal lactoferrin promoted aggregation of the PMN to itself and adherence of the PMN to endothelial cells. This implicates lactoferrin as a regulator of the adherent properties of the PMN during inflammation. They found that anti-lactoferrin antibodies largely inhibited the aggregation responses of PMN to chemotactic stimuli. Therefore it seems likely that lactoferrin rather than any other granule constituent was responsible for the sustained aggregation shown in this study and that lactoferrin has a role to play in retention of PMN at inflammatory sites.

Ambruso et al (1984) studied neutrophil function in a patient

with recurrent pyogenic infections and absence of specific granules. They found that the levels of cobalmin (vitamin B_{12}) -binding protein and lactoferrin were markedly reduced. They also found that the patient's neutrophils were defective in the killing of E. coli and S. aureus, degranulation and adherence were normal, superoxide anion production was normal, but hydroxyl radical production was decreased in response to opsonised zymosan.

This data supports the role of lactoferrin in the bactericidal function of the PMN.

Alkaline Phosphatase

Alkaline phosphatase is the name given to a group of enzymes which hydrolyze phosphoric acid esters at an alkaline pH (pH optimum 9-10). The enzyme contains zinc as an essential component and is effective on a variety of substrates. It has been localised in the specific granules of rabbit PMN by both cytochemical (Wetzel et al, 1967; Bainton and Farquhar, 1968) and fractionation (Baggiolini et al, 1969) techniques. Alkaline phosphatase is associated with the membrane of rabbit specific granules with the catalytic site on the inner face (Bretz and Baggiolini, 1973). In human PMN, cytochemical techniques applied to immature granulocytes suggest the presence of alkaline phosphatase in specific granules (Bainton et al, 1971; Geddes et al, 1975); however, a number of groups have found the alkaline phosphatase of mature PMN in a membrane fraction largely devoid of granules (Bretz and Baggiolini,

1974; Spitznagel et al, 1974; Olsson, 1969). Some enzyme activity is observed in unfixed human neutrophils (Nakatsui, 1969) and in the perinuclear cisternae, Golgi complex and granules of some minimally fixed circulating PMN (Geddes et al, 1975). Thus the localisation of alkaline phosphatase in human PMN remains controversial. It is known however that during neutrophil maturation, alkaline phosphatase activity appears first in the myelocyte stage and gradually increases in more mature forms until it reaches the highest values in the mature neutrophil (Lisiewcz, 1980). Hormonal factors are believed to alter alkaline phosphatase activity (Polishuk et al, 1970).

It is probable that alkaline phosphatase serves a degradative purpose in the PMN rather than acting as a cidal agent.

Other Oxygen-independent Anti-microbial Systems

The enzymes previously mentioned form only a portion of the enzymes and cationic proteins present within the PMN granules. They have been found to possess anti-microbial activity (Miller et al, 1942; Miller and Watson, 1969). This is thought to result from the binding of the highly charged proteins to the negatively charged surface of the organisms, which interferes with the growth and viability of the cells.

pH also has a role in the microbicidal process. During phagocytosis, the hydrogen ion concentration in the phagosome

increases (Mandell, 1970; Jensen and Bainton, 1973). The mechanism of intravacuolar pH depression is unknown, but several possibilities have been proposed: increased lactic acid formation as a consequence of the metabolic burst which accompanies phagocytosis, a redox pump, the presence of H^+ pumping ATPase (Jacques and Bainton, 1978).

Estimates of the pH of phagocytic vacuoles range from 3.0 to 6.0, depending on the species studied (Mandell, 1970; Jensen and Bainton, 1973). Mandell (1970) showed that the fall in the intraphagosomal pH within human PMN is not as great (pH 6.5) as that in macrophages, and it may require 60 minutes to reach this level. Jacques and Bainton (1978) re-investigated this phenomenon and came to the conclusion that the maximal fall in pH *in vivo* and *in vitro* was to pH 5.0, although the pH fall *in vivo* could be lower, they could only demonstrate it to be between 4.0 and 5.0.

It has since been shown that the pH first undergoes an elevation to between 7.8 and 8.0, before slowly falling to about 6.0 over the next few hours (Segal *et al*, 1981). The alkaline phase within the vacuole may be very important for the anti-bacterial activity of cationic granule proteins.

Human PMN contain two types of distinct granules, peroxidase positive azurophils, which also contain the lysosomal enzymes elastase, cathepsin G, chymotrypsin-like cationic proteins and lysozyme, and peroxidase-negative specific granules, which

contain lysozyme, lactoferrin and probably collagenase and vitamin B_{12}-binding protein (Jacques and Bainton, 1981). These substances have pH optima varying over a wide range (Avila, 1977). Both types of granules have been demonstrated to enter the phagocytic vacuole (Bainton, 1973).

The intravacuolar environment changes, which means that the substances within the phagocytic vacuole are subject to changes in pH, with both subsequent activation and/or acid denaturation of its contents. Therefore the pH may have an important role to play in the activation and "switching off" of cidal and degradative enzymes.

Thus the PMN has been endowed with an "overkill" capacity. It contains many enzymes and produces a number of substances which play a role in bacterial killing. The co-operation between oxidative and non-oxidative antimicrobial systems requires further investigation, as the efficient elimination of invading micro-organisms appears to be the result of a synergy between these systems.

1.8 Methimazole And Its Metabclites.

Introduction

Methimazole is one of a group of compounds which exerts anti-thyroid activity, known as the thiourylene drugs. These compounds are used in the treatment of hyper-thyroidism. Methimazole (1-methyl-2-thioimidazole) inhibits thyroid peroxidase, one of the enzymes involved in the biosynthesis

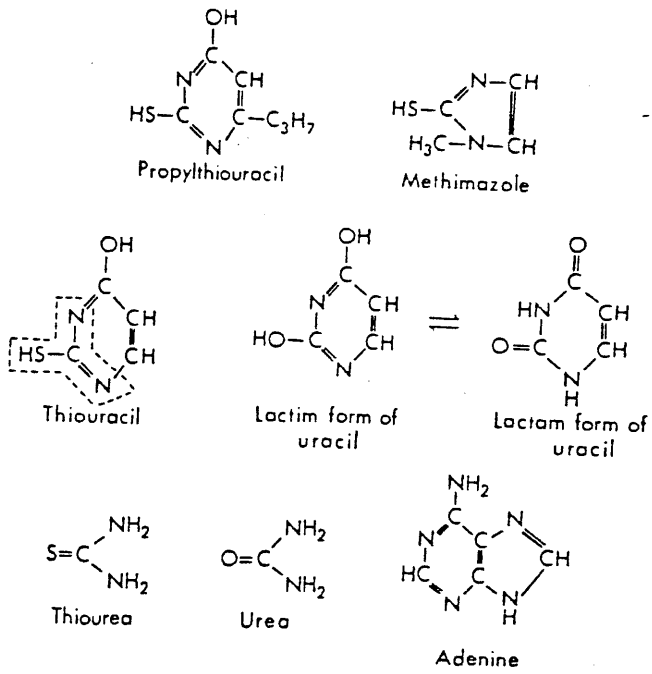


Figure 9. Chemical structures of MMI, PTU and related compounds. The thioureylene group is shown within dashed lines.

of thyroid hormones. The precise mechanism(s) whereby the peroxidase is inhibited is unclear, however, there are a number of theories concerning the mechanism(s) of action, which will be discussed in a later section.

Methimazole is known to be metabolised by rats, (Skellern and Steer, 1981) humans, (Skellern et al, 1977) and more recently by human PMN (Skellern et al, in press). Several of its metabolites have been identified. The PMN contains a number of peroxidases and oxygenases which could potentially be inhibited by methimazole, or one of its metabolites.

Structure And Function Of Methimazole

The majority of organic compounds tested and found to possess anti-thyroid activity, have been of two main types, one containing the thiocarbamide group -NH-C(=S)-, and the other having a heterocyclic or polyhydric phenol type nucleus with one or more polar groups attached such as amino or hydroxyl groups. (Fig 9).

In 1949, methimazole was introduced as an anti-thyroid drug. It was found to have a high degree of anti-thyroid activity, but still exhibited the toxic side-effects of the thiouracils (Stanley and Astwood, 1949). These side-effects include agranulocytosis, rashes, eosinophilia, fever, vasculitis, lupus-like illnesses and neutropenia (McCormick, 1950; Wing and Asper, 1952; Walzer and Einbinder, 1963; Best and Duncan; Amrhein et al, 1970; Cooper et al, 1983; Weitzman and Stossel,

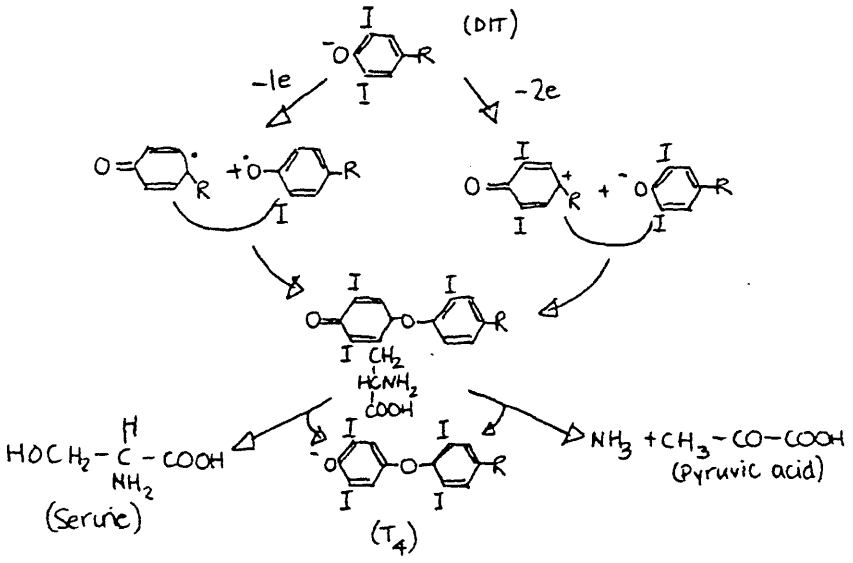
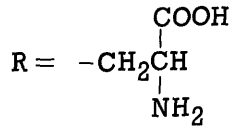


Figure 10. Coupling reactions of DIT to form T_4. Reproduced from De Groot and Niepomniszcze (1977).



1978). These side-effects are uncommon, and there has been little effort made to search for more potent anti-thyroid drugs with fewer toxic side-effects since the 1950's. At present, mainly for commercial reasons, carbimazole, which is rapidly converted to methimazole in human plasma, is the drug of choice in Europe, whereas propylthiouracil and methimazole are used almost exclusively in North America.

Anti-thyroid drugs such as MMI are used in the treatment of hyperthyroidism in three ways: as definitive treatment to control excessive production of thyroid hormone while awaiting a spontaneous remission, before and/or after radio-iodine therapy to hasten recovery while awaiting the effects of irradiation and to control the disorder in preparation for surgical treatment (Marchant et al, 1978).

Mechanism Of Action Of Methimazole

Biosynthesis Of Thyroid Hormones

The thyroid gland is the source of the hormones, thyroxine and triiodothyronine which are essential for normal growth and development, and play an important role in energy metabolism.

A summary of the steps believed to be involved in the biosynthesis and secretion of thyroid hormones is presented in Fig 10 (De Groot and Niepomniszcze, 1977).

The possibility that anti-thyroid drugs worked by inhibiting

thyroid peroxidase activity was suggested even before there was convincing evidence that peroxidase activity plays a role in thyroid hormone formation (Astwood, 1949). With the isolation and purification of thyroid peroxidase (De Groot and Davis, 1962; De Groot et al, 1965; Hosoya et al, 1962; Klebanoff et al, 1962; Igo et al, 1964; Mahoney and Igo, 1964; Hosoya and Morrison, 1967; Coral and Taurog, 1967; Taurog et al, 1970; Pommier et al, 1972), it became clear that most compounds with anti-thyroid activity are inhibitors of thyroid peroxidase-catalysed iodination.

Mechanism Of Action Of The Thiourylene Drugs

There have been several proposals for the precise mechanisms by which anti-thyroid compounds inhibit thyroid peroxidase catalysed reactions. Morris and Hager (1966) proposed a mechanism based on studies with a mould chloroperoxidase, which catalysed halogenation at acid pH. They found that chloroperoxidase catalysed the oxidation of thiouracil only in the presence of iodide or another halogen, and concluded that tyrosine and thiouracil were competitive substrates for a common reactant. They proposed the formation of a peroxidase-iodinium complex ($E\text{-}I^+$) which could act as an iodinating agent towards tyrosine, or as an oxidising agent towards thiouracil. According to this hypothesis anti-thyroid drugs of the thiourylene type inhibit iodination by competing with tyrosine or tyrosyl residues of thyroglobulin for $E\text{-}I^+$.

Maloof and Soodak (1965) proposed a similar mechanism based on their observation that thiourea is oxidised by crude thyroid peroxidase in the presence, but not in the absence of iodide. This implies that the thiourylene drugs should inhibit only peroxidase activity involving iodide oxidation. However, it is known that MMI, PTU and carbimazole are also potent inhibitors of thyroid-peroxidase (TPO) -catalysed oxidation of guaiacol, a reaction that does not involve iodide, as well as inhibiting TPO-catalysed iodination of proteins and tyrosine. Therefore the thiourylene drugs must be considered to act as general inhibitors of TPO-catalysed reactions and not as specific inhibitors of TPO-catalysed iodination.

Nagasaka and Hidaka (1976), in a study using partially purified human TPO concluded that the mechanism of PTU inhibition is essentially different from that of MMI, the former being reversible, the latter irreversible. However, Taurog (1976) proposed a scheme in which both PTU and MMI display reversible or irreversible inhibition depending on the relative concentrations of drug and iodide. The main points of the scheme were that at high drug to iodide concentrations, the inhibition of iodination was irreversible, at low ratios of drug to iodide concentration, iodination was not inhibited. Iodide antagonised inhibition by low drug concentrations, but not by moderately elevated drug concentrations, at low iodide concentrations iodination was more sensitive to inhibition. Increased tyrosine concentrations

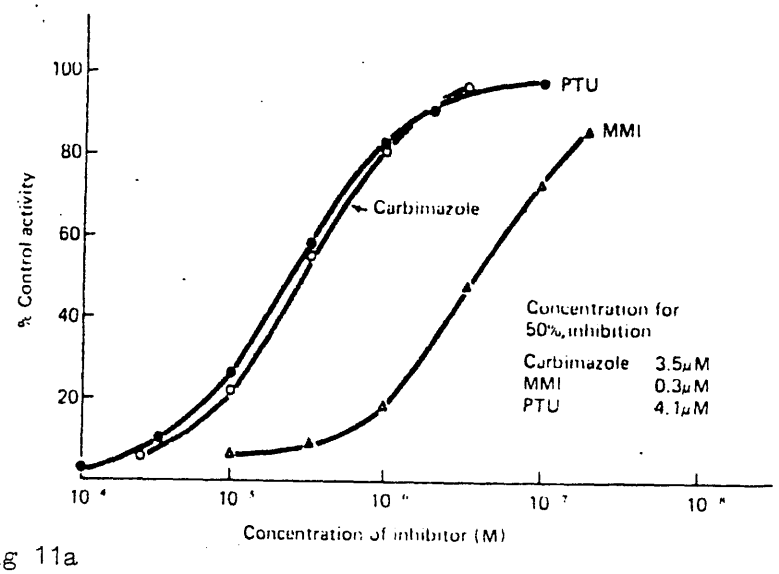


Fig 11a

Low ratio of drug to iodide	Increased ratio of drug to iodide
Inhibition by drug 1 $E + I^- + H_2O_2$ → [E-I] Activated iodine	Inhibition by drug 1. $E + I^- + H_2O_2$ → [E-I] Activated iodine
2. [E-I] → Drug → Oxidized drug (preferred pathway) [E-I] → Tyrosyl → Iodinated tyrosyl	
A. Inhibition of reaction 1 by drug largely overcome by iodide	A. Reaction 1 largely blocked by drug
B. [E-I] formation occurs readily	B. Very low formation of [E-I]
C. Extensive drug oxidation	C. Little or no drug oxidation
D. Escape of iodination from inhibition after lag period	D. No escape of iodination from inhibition

Fig 11b

Figure 11a shows dose-inhibition curves for inhibition by thioureylene drugs of TPO-catalyzed oxidation of guaiacol. Incubation tubes contained 1 μg/ml TPO, 1 mM guaiacol, 0.5 mg/ml BSA, and 100μM H_2O_2. The reaction was initiated with H_2O_2 and A_{470} was read after 1 min at 25°C. (Taurog, 1976).

Figure 11b shows proposed scheme for mechanism of inhibition by thiourylene drugs of TPO-catalyzed iodination. (Taurog, 1976).

could not overcome inhibition by low drug concentrations. MMI and PTU were capable of inhibiting their own and each others oxidation. A summary of the scheme is shown on Fig.11a,b.

It was subsequently shown by Davidson et al (1978) that thiourylene drugs could irreversibly inactivate TPO in the presence of H_2O_2 (a reaction not considered by Taurog et al), providing an explanation for the irreversible inhibition of TPO-catalysed iodination. Engler et al (1982), using a more highly purified TPO also investigated this mechanism and concluded that the inactivation of TPO by MMI and PTU involves a reaction between the drugs and the oxidised haem group produced by an interaction between TPO and H_2O_2. This conclusion was supported by the observation that addition of a low concentration of H_2O_2 to a sclution of TPO shifted the λ_{max} of the Soret band from 411 to 420nm, reflecting the formation of an oxidised form of TPO (TPO_{ox}). Addition of MMI or PTU to TPO_{ox} produced a Soret spectrum significantly different from the spectrum of native TPO or TPO_{ox}, whereas addition of MMI or PTU to native TPO produced no significant change in the haem spectrum. Studies with radiolabelled MMI and PTU combined with simultaneous assays of enzyme activity (guaiacol assay) showed that firm binding of the drugs to TPO and inactiviation of the enzyme occurred on addition of the drugs to TPO_{ox}. However, neither binding not inactivation occured on addition of the drugs to native TPO. Lastly, the presence of a low concentration of iodide prevented the shift in the Soret spectrum, binding of labelled drug,

and loss of enzyme activity associated with the addition of thiourylene drugs to TPO + H_2O_2. Under these conditions they assumed the enzyme was present as TPO.I_{ox}, a form in which the haem is present in the same reduced state as native TPO, explaining the protective action of iodide on the inactivation of TPO_{ox} by MMI and PTU.

Inactivation of lactoperoxidase (LPO) by thioureylene drugs was studied by Edelhoch and co-workers (1979). They studied LPO-catalysed oxidation of N-acetyltyrosylamide at pH 8.8, measuring rate constants for oxidation of thioureylene drugs by LPO as well as for inactivation of LPO by the same drugs. The influence of iodide on both rate constants was studied to distinguish between the roles of iodide in iodination and drug oxidation. They concluded that iodide inhibited goitrogenic activity either by increasing the rate of drug oxidation or by reducing the rate of enzyme activation, or both, depending on the particular drug. The mechanism by which the thioureylene drugs inhibit peroxidase-catalysed iodination was not explicitly discussed, leaving the implication that they inhibit iodination solely by inactivating the peroxidase. Edelhoch et al (1979) also suggested that iodide was bound to a regulatory site on the peroxidase and that oxidation of iodide was not involved in drug oxidation. Engler et al (1983) further investigated the role of iodide in overcoming inhibition of iodination by thioureylene drugs and in contrast to Edelhoch and co-workers proposed an oxidising iodine species as an intermediate in TPO-catalysed oxidation of thioureylene drugs. They updated their previous

scheme for the mechanism of reversible and irreversible inhibition of TPO-catalysed iodination by thioureylene drugs, (Fig. 12). Reaction 1 shows inactivation of the native form of TPO by thioureylene drugs, but in the presence of H_2O_2 this is a minor pathway (dotted lines) (Engler et al, 1982). Reaction 2 shows the oxidation of native TPO by H_2O_2 to yield the functional form of the enzyme, designated TPO_{ox}. The major difference between this scheme and the previously reported one (Taurog, 1976) is that the revised scheme shows the thioureylene drugs can irreversibly inactivate TPO (Reaction 3). Reaction 4 shows inactivation of TPO_{ox} by excess H_2O_2; the dashed lines indicate that the reaction is minor to reaction 3. Reaction 5 shows the oxidation of I^- by TPO_{ox} to form oxidised iodine. It is likely that the active iodinating agent is a complex between TPO and oxidised iodine ($TPO\text{-}I_{ox}$) (Taurog, 1976). Evidence by Ohtaki et al (1981) suggests that TPO-catalysed oxidation of I^- involves a two electron change, if this is the case then the active iodinating agent in TPO-catalysed iodination should be written as $TPO\text{-}I^+$. The formation of an intermediate oxidation product in the reaction between $TPO\text{-}I_{ox}$ and drug is indicated in reaction 6, there is evidence to suggest that the same intermediate is formed under conditions of both reversible and irreversible inhibition of iodination. Since no metabolic product was observed in the absence of iodide, and since sulphenyl iodide has been implicated as an intermediate in the oxidation of thioureylene drugs by iodide (Davidson et al, 1978; Morris and Hager, 1966; Danehy, 1971). Engler et al assumed

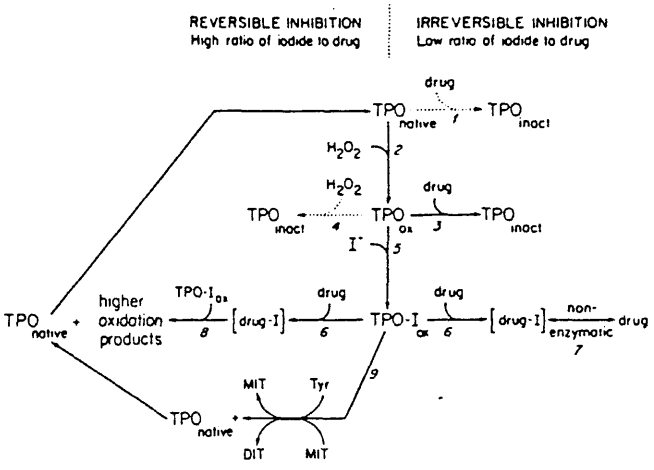


Figure 12. Proposed scheme for mechanism of inhibition of TPO-catalyzed iodination by thioureylene drugs (Engler et al, 1982).

that the spectrally determined early stage intermediate, shown in reaction 6, might be the sulphenyl iodide. This intermediate, whatever its nature appears in the case of MMI to be able to reform the parent compound (reaction 7). This does not appear to be the case with PTU. The metabolism of the thioureylene drugs that occurs under conditions of irreversible inhibition (reaction 6) is very limited. Oxidation of MMI and PTU beyond the intermediate stage (reaction 6) requires a relatively high concentration of iodide, this is shown in reaction 8 as a part of the scheme leading to higher oxidation products and reversible inhibition of iodination. Under these conditions the rate of production of TPO-I_{ox} is sufficient to further oxidise the drugs before the TPO is largely inactivated in reaction 3. Higher oxidation products of both MMI and PTU have been identified both *in vivo* (Marchant and Alexander, 1972; Pharmakiotis and Alexander, 1975; Nakashima *et al*, 1978; Lindsay *et al*, 1979) and *in vitro* (Taurog, 1976). Sulphate is a major thyroidal end-product of PTU and MMI oxidation (Taurog, 1976; Marchant and Alexander, 1972; Nakashima *et al*, 1978).

The basic assumption underlying this mechanism is that both iodination and drug oxidation are mediated by the same enzyme-iodine complex (TPO-I_{ox}). Drug oxidation is the preferred pathway when MMI or PTU competes with tyrosyl residues for oxidised iodine, and as long as sufficient drug is present, TPO-I_{ox} will be diverted from iodination to drug oxidation. After the drug concentration is reduced to a low level, TPO-I_{ox}

will be used to iodinate tyrosyl residues. Native TPO is regenerated during both oxidation and iodination.

It is well accepted that TPO catalyses both iodination and coupling in the thyroid gland. The thioureylene drugs are known to inhibit TPO-catalysed iodination. Richards and Ingbar (1959) suggested that PTU may have a specific inhibitory effect on the coupling reaction, independent of its effect on iodination. Taurog (1970) apparently confirmed this with a series of experiments in which graded doses of PTU were added to a TPO model iodination system, however these experiments had a number of short comings which were remedied by Engler et al, (1982a). They showed that under certain conditions of incubation, it was possible to demonstrate a significant inhibitory effect on T_4 and T_3 formation without any decrease in diiodotyrosine formation. Their observations indicate that, at least under some conditions PTU and MMI can exert a specific inhibitory effect on the coupling reaction. In the case of PTU, a specific inhibitory effect on coupling was also demonstrated with an incubation system in which TPO-catalysed coupling was measured in the absence of iodination.

The mechanism of TPO and LPO-catalysed iodination of tyrosine and tyrosyl residues in protein has been discussed. According to these schemes it would be expected that one mol. of H_2O_2 is required for the organic binding of one mol. of iodide. Magnusson et al (1984) examined the stoiciometric relationship

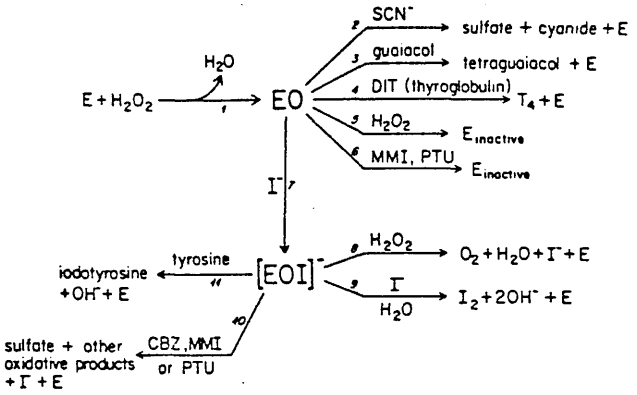


Figure 13. Proposed scheme to explain iodide-dependent catalatic activity of thyroid peroxidase and lactoperoxidase, showing relationship of this activity to peroxidative functions of the enzymes (represented in scheme as E). (Magnusson et al, 1984).

between organic iodide formation and H_2O_2 utilisation in iodination reactions catalysed by TPO and LPO. Iodination of tyrosine, bovine serum albumin, or low iodine thyroglobulin was carried out under conditions where H_2O_2 was limiting. The ratio of moles of iodide bound per mol. of H_2O_2 used was always less than 1.0. All the H_2O_2 disappeared from the reaction mixture, indicating that H_2O_2 was being used in some reaction other than iodination. Magnusson et al (1984) proposed that TPO and LPO possess catalase-like activity, this catalatic activity was established by demonstrating that O_2 is readily evolved from H_2O_2 by TPO and LPO. This activity was completely iodide dependent. It is believed that iodide is catalytically involved in the enzymatic reaction leading to the H_2O_2 disappearance. (Fig 13)

This reaction may be inhibited reversibly or irreversibly by MMI, depending on the concentration of the drug. They have postulated that oxidation of I^- by compound I of the peroxidases to form enzyme-bound hypoiodite. They suggest that this compound can react with H_2O_2 in a catalase-like reaction with evolution of O_2. This same form of oxidised iodine would be involved in the iodination of tyrosine, oxidation of the thioureylene drugs and oxidation of I^- and that inhibition of catalatic activity by these agents occurs through competition with H_2O_2 for oxidised iodine.

Methimazole Pharmacology In Man

Little was known of MMI pharmacology until relatively recently when isotopic (Alexander et al, 1969; Marchant and Alexander, 1972; Papapetrou et al, 1972; Sitar and Thornhill, 1973; Skellern et al, 1973), colorimetric (Pittman et al, 1971; Vessell et al, 1975), chromatographic and mass spectroscopic (Skellern et al, 1976; Bending and Stevenson, 1978; Floberg et al, 1980) methods were devised to measure MMI concentrations in body fluids. Cooper et al (1984) developed a radio-immunoassay for MMI. A MMI derivative, 5-COOH-MMI, was conjugated to porcine thyroglobulin and antibodies to the conjugate were raised in rabbits. Radiolabelled (^{35}S-) MMI was used as the tracer, the assay could detect MMI in amounts as low as 2.5ng. The putative MMI metabolites 3-methyl-2-thiohydantoin and 1-methyl-imidazole had minor cross-reactivities of 2.1% and 0.5% respectively. They found considerable inter-individual variation in peak serum MMI levels, which may have been due to differences in drug absorption or metabolism. It has been reported that there is almost complete absorption of MMI from the gastrointestinal tract (Shimmins, 1969).

Peak serum levels of MMI occurred after about 1-2 hours, with a second, smaller peak of MMI immunoactivity occuring after 4-6 hours in 80% of human subjects (Cooper et al, 1984). Similar observations have been made with carbimazole, but

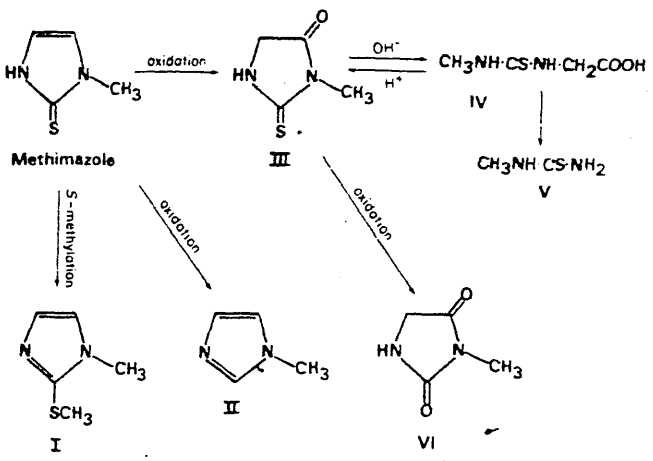


Figure 14. Proposed metabolic pathways of (2-^{14}C) MMI metabolism. Reproduced from Skellern and Steer, 1981).

the phenomenon remains to be explained (Skellern et al, 1980). The serum half-life for MMI was found to vary slightly between investigators. Cooper et al (1984) found it to be around six hours, Johansen et al (1982) found it to be around five hours, and several other investigators three to four hours (Skellern et al, 1974, 1980, 1980b; Melander et al, 1980; Hallengren et al, 1982). Vessell et al (1975) found by colorimetric methods, an increase in half-life in hyperthyroid patients, Hallengren et al (1982) and Cooper et al (1984) found no differences in the serum half-life between euthyroid and hyperthyroid individuals, in contrast, the serum half-life was prolonged in patients with hepatic disease suggesting a role for the liver in MMI metabolism.

Williams et al (1944) recommended that breast-feeding should be avoided by mothers taking any antithyroid drugs. It has been found that the mean serum and milk MMI concentrations were equal over an 8-hour period, with 70 ug MMI passing into the milk after a single dose of 40-mg (Cooper et al, 1984). Johansen (1982) found a mean milk to serum ratio of 0.98. PTU is one tenth as potent as MMI, therefore the daily dosage taken is ten times that of MMI, however it crosses into milk one tenth as well as MMI with the net result that PTU is the drug of choice where a mother chooses to breast feed, during which time the baby's thyroid function would be monitored.

Metabolism Of MMI In Man And Animals

TPO oxidises MMI (Taurog, 1976; Nakashima and Taurog, 1979).

3-methyl-2-thiohydantoin (3-MTH), has been detected in the thyroid, in plasma and in the urine of patients treated with MMI or carbimazole (Skellern et al, 1977, 1980a, 1981). The biologic half-life of this metabolite is 13.5 hours. Whether the metabolites of antithyroid drugs still exert anti-thyroid effects in man is unknown. 3-MTH has antithyroid activity in rats (Searle et al, 1951), but apparently not in humans (Alexander et al, in press).

N-methylimidazole (MI) and 3-MTH have been identified as metabolic products when MMI was incubated with rat liver microsomes (Lee and Neal, 1978). When MMI was incubated with a pig liver microsomal flavoprotein mixed-function oxidase (Poulsen et al, 1974), N-MI and sulphite were identified as oxidation products. MMI has been shown to be S-methylated in the presence of a purified rat liver S-methyltransferase (Weisiger and Jakoby, 1979).

Skellern and Steer (1981) proposed a metabolic pathway for (2-^{14}C) MMI metabolism (Fig 14). Their study confirmed that after chronic intraperitoneal administration of (2-^{14}C) MMI to rats (17.6mg/kg for three days), the radioactivity is rapidly and appreciably eliminated in the urine and that MMI is extensively metabolised, since only 6.7% of the dose was excreted unchanged. Six solvent-extractable metabolites were isolated and characterised as N-methylimidazole,

3-methyl-2-thiohydantoin, 1-methyl-2-thiohydantoic acid, N-methylthiourea and a methylhydantoin.

After ^{14}C-MMI and ^{35}S-MMI administration, the ratio of tissue to plasma concentration is less than one in all tissues except for the thyroid, liver, kidney and salivary gland (Marchant et al, 1972a; Ferguson et al, 1984). Maximal concentration in the thyroid is reached after 24 hours, in liver and kidney after 6 hours (Skellern et al, 1973). A significant percentage of circulating drug radioactivity is contained within the blood cells (Marchant and Alexander, 1972b).

The Effect Of MMI On Granulocytes.

An uncommon side effect on treatment with antithyroid drugs is agranulocytosis, the prevalence is not known. Agranulocytosis is most common in PTU treatment, and it has been suggested that it is a hypersensitivity reaction (Wall et al, 1984). These patients may develop agranulocytosis as a result of underlying immunologic abnormalities. Because there is bone marrow depression in these patients, the immunologically-mediated damage presumably affects the stem cells rather than mature, circulating neutrophils. It is thought that the thioamide group of anti-thyroid drugs acts as the antigen or becomes antigenic by forming a complex with granulocyte stem-cell protein. This complex may act as an adjuvant to the immune system, with bystander neutrophil damage (Schoen and Trentham, 1981). Another possibility is

that antibody-dependent killer lymphocyte-mediated cytotoxicity against sensitized granulocytes may be a mechanism for anti-thyroid drug-associated agranulocytosis.

Antithyroid drugs also have a well established association with severe neutropenia (Huguley, 1964; McGavack and Chevalley 1954). Weitzman and Stossell (1978) showed neutrophils opsonised by a complement-dependent mechanism apparently initiated by an IgM antibody in a patient treated with MMI. They proposed that neutrophil destruction occurs in patients like this by sequestration and phagocytosis of opsonized cells by macrophages, as has been shown in spleens of patients with autoimmune neutropenia (Boxer et al, 1975).

The Effect Of MMI On PMN

Although PMN are vastly different from thyroid cells in primary function, both are able to concentrate and metabolise iodide (Stolc, 1971). It seemed likely that the PMN would also be capable of accumulating antithyroid drugs. Lam and Lindsay (1979) showed this to be the case. Phagocytosing PMN accumulate radioactive (^{14}C)-PTU and MMI at rates eight to ten fold those of resting cells. However this accumulation was independent of iodide transport and metabolism which is in contrast to that observed in the thyroid. These findings suggested that the PMN may be another important site for the metabolism and disposition of MMI and may exert their biological effects.

MMI inhibits iodination by the thyroid gland; Klebanoff (1967) showed that it also inhibits iodination by phagocytosing PMN. MMI inhibits iodide organification, but not iodide transport (Lam and Lindsay, 1980). It was shown that iodide uptake by phagocytosing PMN was not affected by MMI, or by the classical inhibitors of active anion transport such as perchlorate or antimycin A at concentrations producing over 90% inhibition in thyroid cells. Therefore it was concluded that iodide uptake in the PMN is not an active process (Lam and Lindsay, 1980; Ferguson et al, 1984).

The antimicrobial activity of myeloperoxidase and H_2O_2 is considerably increased by the addition of a co-factor such as a halide, of the co-factors tested, iodide is the most effective (Klebanoff, 1967, 1968). Complete killing of microorganisms was observed at a concentration of 5×10^{-6}M (60ug%). The inorganic iodide concentration of serum is very low (1ug%) and it is unlikely that sufficient iodide can enter the cell from this source to produce a significant microbicidal effect in vivo (Lam and Lindsay, 1980). The thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) can replace iodide as the co-factor in the isolated MPO-mediated antimicrobial system (Klebanoff, 1967). The hormones are de-iodinated by MPO and H_2O_2 under these conditions (Klebanoff and Green, 1973). The thyroid hormones bind to intact leukocytes (Roche et al, 1962; Siegel and Sachs, 1964; Klebanoff and Hamon, 1972). T_4 and T_3 are degraded by intact,

phagocytosing PMN with the release of the iodine as inorganic iodide (Woeber et al, 1972; Klebanoff and Hamon, 1972; Klebanoff and Green, 1973; Woeber and Ingbar, 1972). Rao and Sagone (1984) postulated that a reactive oxygen species such as a chloramine produced by the MPO-H_2O_2-halide system was responsible for the oxidation and fixation of iodide to protein. However they found that PMN stimulated by opsonised zymosan particles were capable of carrying out the iodination of T_3 to T_4 in the presence of excess I^-, this makes the availability of extra iodide being present for the MPO-H_2O_2-halide system questionable. Therefore although the MPC-H_2O_2-I^- system is inhibited by MMI, its relevance in vivo is doubtful.

In early studies by Klebanoff and White (1969) MMI did not significantly affect the killing of L. acidophilus by normal leukocytes, but consistently decreased the rate of killing of this organism by chronic granulomatous disease leukocytes. MMI was subsequently found to have a small but significant inhibitory effect on the staphylocidal activity of normal leukocytes (Klebanoff, 1972; Klebanoff and Hamon, 1972). The antithyroid drug PTU also inhibits iodination and the killing of staphylococci by human PMN (Klebanoff and Hamon, 1972). MMI did not affect the phagocytosis or viability of any organism tested by Klebanoff (Klebanoff, 1969; Klebanoff and Hammon, 1972). Ferguson et al (1984) similarly found phagocytosis of opsonised zymosan unaffected by MMI or PTU.

Resting PMN from PTU-treated rats have been shown to have increased rates of oxygen consumption, glucose uptake, lactate production, glucose-1-^{14}C oxidation and glucose-6-^{14}C oxidation and the increment in oxygen uptake and glucose-1-^{14}C oxidation during phagocytosis also was increased (Reed and Tepperman, 1969). PTU, MMI, thiouracil and thiourea were found to stimulate glucose-1-^{14}C oxidation by phagocytosing human PMN in vitro (Tsan and McIntyre, 1975).

Resting hexose monophosphate shunt activity was increased by PTU only in the presence of added H_2O_2, suggesting a dependence on H_2O_2 for the stimulation observed with phagocytic cells.

Lehrer (1975) tested the ability of human monocytes to kill Candida albicans and Candida parapsilosis in the presence of MMI, he found it was unaffected by concentrations of 1mM MMI, although iodination was inhibited.

Weetman et al (1984) using measurement of luminol-dependent chemiluminescence as an assay for oxygen radical production, found that in stimulated, human monocytes, MMI inhibited oxygen radical production. Ferguson et al (1984) found a similar effect in human PMN. They postulated this to be as a result of MMI inhibiting the cells' peroxidase/oxygenase system.

Thus MMI has been implicated as having a role in the PMN, however further information is required to assess the significance of this role.

CHAPTER 2

THE EFFECT OF METHIMAZOLE AND SOME OF ITS PROPOSED METABOLITES ON THE CHEMOTACTIC RESPONSE OF HUMAN PMN TO ZYMOSAN-ACTIVATED SERUM.

2.0 Introduction.

Chemotaxis denotes the directional movement of motile cells in response to a chemical gradient and is thought to account for the accumulation of granulocytes and lymphoid cells at sites of tissue injury or infectious invasion. In these situations, the primary attracting agent may be activated complement, chemical factors released by the pathogen, and/or altered products of other host or microbial components (Wilkinson, 1980).

Thus chemotaxis can be thought of as the primary response of PMN to invading pathogens, being the first step towards their phagocytosis and ultimate destruction. Thus it is necessary to know whether methimazole or any of its proposed metabolites affect the chemotaxis of human PMN.

2.1 Materials And Methods.

Isolation Of Granulocytes

Venous blood was withdrawn, at the same time each morning, between 9 and 10 a.m. from healthy, human, male volunteers,

aged between 20 and 30 years. The blood was immediately mixed with preservative-free heparin in phosphate buffered saline (PBS). The blood was mixed with Plasmagel (Laboratoire Roger Bellon) in a ratio of 1 part Plasmagel to 2 parts blood, and the red blood cells were left to sediment at 37°C for 30 minutes. The top layer containing leukocytes and plasma was withdrawn and any remaining erythrocytes were hypotonically lysed and the granulocytes were washed and resuspended in Minimal Essential Medium (MEM) to give a concentration of 10^7 cells per ml.

The cell preparations were stained with Wright's stain (Gurr), and contained on average 95% PMN. Viability was checked by trypan blue exclusion.

Preparation Of Chemotactic Factors

E.coli Supernatant

A dense innoculum of E.coli (clinical strain) was put into 80 ml Medium 199 (Gibco Ltd), and incubated at 37°C for 30 hours. The culture was spun at 1,500 g for 10 minutes, and the supernatant was removed and filtered through 0.22 um filters. The filtered supernatant was aliquotted into sterile, plastic bijoux bottles and stored at -20°C.

Normal Human Serum

Twenty ml of venous blood was withdrawn from healthy, human

volunteers in sterile syringes and transferred to glass clotting bottles, the blood was allowed to clot at room temperature and was then spun at 600 g. The serum was pipetted off and filtered through 0.22 μm millipore filters, and used fresh as a chemo-attractant.

Zymosan Activated Serum

50 ug per ml zymosan A (Sigma) was added to fresh human serum. The mixture was incubated at 37°C for 30 minutes with end-over-end rotation, and spun at 2,000 g for 5 minutes to sediment the zymosan. The serum was removed and used fresh or was stored at -20°C.

Preparation Of Agarose Plates

The plates were prepared according to the method of Nelson et al (1975). Solution A was prepared as follows: 750 mg of agarose (Gibco) was dissolved in 50 ml of double distilled water in a boiling water bath for 10 to 15 minutes. Solution B consists of 10 ml of 10 X MEM, 10 ml of heat-inactivated fetal calf serum (Gibco), 1 ml of glutamine (Gibco), 1 ml of Pen/Strep containing 100 U per ml penicillin and 100 μg per ml streptomycin (Gibco), 0.09 g $NaHCO_3$ and 28 ml of double distilled water. This was warmed to 48°C.

Solutions A and B were mixed together and aliquotted into tissue culture dishes (60 X 15 mm) in 5 ml amounts. The

plates were refrigerated for 30 to 60 minutes, then using a template, wells were cut in the plates.

Chemotactic Assay

The wells of the agarose plates were loaded with 10 µl of cells, chemotactic factors or MEM. The inside wells received aliquots of cells, and the outside wells received either chemotactic factor or MEM as a control.

The plates were incubated in a moist CO_2 incubator at 37°C for 4 hours. The cells were fixed by the addition of 3 ml of methanol to each plate, which were left for 30 minutes followed by an equal volume of formal saline for 30 minutes. The agarose was then removed with a spatula and the plates stained with either filtered Wright's or Giemsa stain and air dried. The plates were examined under a microscope and chemotaxis was quantified, using an ocular micrometer, in arbitrary units.

The Effect Of Methimazole And Its Putative Metabolites On The Chemotaxis Of Human PMN

Methimazole (Sigma), 3-methylthiohydantoin (a gift from Dr G.G. Skellern), methylhydantoin (Sigma), and N-methylimidazole (Sigma) were dissolved in sterile saline to give final concentrations of 10^{-5} to 10^{-3} M when added to the PMN suspensions. The chemotaxis assay was conducted as above.

All the assays were performed in triplicate.

2.2 Results.

Comparison Of Chemoattractants And Incubation Times

The chemotactic assay was carried out to compare the chemoattractants: normal human serum, zymosan activated serum and E.coli supernatant. The assay was also carried out with incubation times of two and four hours. The chemoattractants varied in potency, with zymosan activated serum giving the greatest chemotactic index (the linear distance the cells have moved from the margin of the well towards the chemotactic factor (distance A) over the linear distance the cells have moved from the margin of the well towards the control medium (distance B)), and chemotactic differential (distance A-B). Normal human serum was next best and E.coli supernatant was the least potent of the three.

It was found that an incubation time of four hours resulted in a greater amount of locomotion than two hours. Incubation times of longer than four hours did not result in a greater amount of chemotaxis.

The Effect Of Methimazole (MMI), Methylthiohydantoin (MTH), Methylhydantoin (MH) And Methylimidazole (MI) On The Chemotactic Response Of Human PMN To Zymosan-activated Serum

The results indicate that MMI, MTH, MH and MI do not affect

Table 1

Table of linear distance travelled by PMN away from (B) and towards (A) the control substance MEM (minimal essential medium). Correlation Coefficient statistics were performed on the chemotactic index (A/B) and chemotactic differential (A-B) results. No significant dose-related effect on random movement of PMN was found as a result of treatment with any of the tested drugs. The mean values presented represent the average of 3 separate determinations performed in triplicate.

TABLE 1

Effect Of MMI, MTH, MH And MI On The Random Movement Of Human PMN

Drug	Log Molar Conc	$\bar{x}A \pm s$	$\bar{x}B \pm s$	$\bar{x}A/B \pm s$	$\bar{x}(A-B) \pm s$
MMI	0	2.00 ± 0.50	1.67 ± 0.29	1.22 ± 0.39	0.33 ± 0.58
	-3	1.92 ± 0.14	1.75 ± 0.25	1.11 ± 0.19	0.17 ± 0.29
	-4	1.92 ± 0.52	1.75 ± 0.25	1.22 ± 0.39	0.17 ± 0.29
	-5	2.00 ± 0.43	1.75 ± 0.00	1.14 ± 0.25	0.25 ± 0.43
MTH	0	1.67 ± 0.29	1.67 ± 0.29	1.00 ± 0.00	0.00 ± 0.00
	-3	1.83 ± 0.29	1.67 ± 0.29	1.11 ± 0.19	0.17 ± 0.29
	-4	1.83 ± 0.29	1.75 ± 0.25	1.05 ± 0.08	0.08 ± 0.14
	-5	2.00 ± 0.50	2.00 ± 0.50	1.00 ± 0.00	0.00 ± 0.00
MH	0	2.50 ± 1.00	1.67 ± 0.29	1.47 ± 0.41	0.83 ± 0.76
	-3	2.17 ± 0.29	1.67 ± 0.29	1.33 ± 0.34	0.50 ± 0.50
	-4	1.92 ± 0.14	1.58 ± 0.14	1.22 ± 0.19	0.33 ± 0.29
	-5	1.67 ± 0.29	1.67 ± 0.29	1.00 ± 0.00	0.00 ± 0.00
MI	0	1.75 ± 0.25	1.75 ± 0.25	1.00 ± 0.00	0.00 ± 0.00
	-3	1.92 ± 0.14	1.58 ± 0.14	1.22 ± 0.19	0.33 ± 0.29
	-4	1.92 ± 0.14	1.92 ± 0.14	1.00 ± 0.00	0.00 ± 0.00
	-5	2.00 ± 0.66	1.58 ± 0.14	1.28 ± 0.48	0.42 ± 0.72

Table 2

Table of linear distance travelled by PMN away from (B) and towards (A) zymosan-activated serum. Correlation Coefficient statistics were applied to the results, no significant dose-related effect on chemotaxis or chemokinesis was found.

The results represent the means of 3 separate determinations performed in triplicate.

TABLE 2

Effect Of MMI, MTH, MH And MI On The Chemotaxis And Chemokinesis Of Human PMN Using Zymosan-activated Serum As Chemo-attractant

Drug	Log Molar Conc	xA±s	xB±s	xA/B±s	x(A-B)±s
MMI	0	4.33±0.29	1.50±0.00	2.89±0.19	2.83±0.29
	-3	3.67±1.15	1.50±0.00	2.44±0.77	2.17±1.15
	-4	3.58±0.52	1.50±0.00	2.39±0.35	2.08±0.52
	-5	3.67±0.58	1.67±0.29	2.22±0.39	2.00±0.50
MTH	0	3.83±0.29	1.50±0.00	2.56±0.20	2.33±0.29
	-3	3.67±0.29	1.50±0.00	2.44±0.20	2.17±0.29
	-4	4.17±1.15	1.67±0.29	2.58±0.98	2.50±1.32
	-5	3.67±0.29	1.67±0.29	2.25±0.47	2.00±0.50
MH	0	3.67±0.29	1.50±0.00	2.44±0.20	2.17±0.29
	-3	4.83±0.58	1.83±0.29	2.72±0.82	2.67±0.29
	-4	4.75±0.66	1.50±0.00	3.17±0.44	3.25±0.66
	-5	4.33±0.29	1.50±0.00	2.89±0.19	2.83±0.29
MI	0	4.33±0.29	1.67±0.29	2.67±0.58	2.67±0.58
	-3	4.50±0.87	1.83±0.29	2.56±0.96	2.67±1.15
	-4	4.50±0.00	1.58±0.14	2.86±0.25	2.92±0.14
	-5	4.33±0.29	2.08±0.14	2.57±0.55	2.25±0.43

chemotaxis. (Table 2) The chemotactic index and differential was calculated for each drug and correlation coefficient statistics applied to these data. (Table 2)

The chemotactic index for the PMN moving away from and towards the control substance (MEM) was approximately 1.0, in the presence or absence of all four drugs, indicating that there was no directed movement towards the control substance. This gives a measure of the amount of random migration by the PMN in a given period of time. The chemotactic index for the PMN travelling towards zymosan-activated serum was between 2.5 and 3.0, indicating that there was no significant difference between the chemotactic index of the cells in the presence and absence of MMI, MTH, MH or MI.

Thus, there was a significant amount of directed movement by the PMN towards zymosan-activated serum, a recognised chemotactic factor. However, this movement was unaffected by the presence of any of the tested drugs.

Controls were included to establish that the drugs themselves did not act as attractants or repellents to the PMN.

2.3 Discussion.

In the absence of a stimulus, PMN display a slow level of random movement. A number of chemical stimuli increase the

motility of PMN. When stimuli act in the absence of a concentration gradient, the rate and degree of random motility is enhanced. This chemically enhanced non-directional locomotion is referred to as chemokinesis. When the PMN are exposed to a concentration gradient of the chemical stimulus, the cells respond by moving towards or away from the stimulus. This directed locomotion is known as chemotaxis, and accounts for the accumulation of granulocytes and macrophages/monocytes at sites of tissue injury or infectious invasion. It is therefore, *in vivo* the important first step towards elimination of invading micro-organisms.

A comparison of chemotactic factors was made, namely *E.coli* supernatant, normal human serum and zymosan-stimulated serum. *E.coli* produces N-f-mlp as its major peptide neutrophil chemotactic factor (Marasco *et al*, 1983), human serum contains chemotactic factors, but on stimulation of the classical or alternative complement pathways by eg. zymosan, a number of chemotactically active compounds are produced, the most important of which is C5a, and it was found to be the strongest attractant with normal human serum next and the *E.coli* supernatant weakest.

Four hours was chosen as the incubation time for the assay, as no significantly greater amount of chemotaxis was observed with incubation times of greater than four hours. PMN have

a limited lifespan *in vitro*, and their activity deteriorates rapidly after isolation. It is possible that following the lengthier incubation times suggested by some authors, the cells have become extremely sluggish or non-viable and thus no greater amount of chemotactic activity is demonstrated.

Many different compounds have been shown to affect chemotaxis in a number of different ways, therefore it was important to assess the effect of MMI and some of its putative metabolites on human PMN chemotaxis. A number of imidazole compounds were tested by Rowan-Kelly *et al* (1984), who found that metrinidazole and tinidazole did not affect chemotaxis, but clotrimazole, miconazole and ketoconazole inhibited human PMN chemotaxis and depressed their ability to kill bacteria and fungi. The authors suggested that these imidazoles interfere with lipid biosynthesis and exert their effects by causing changes in the PMN cell membrane (Van den Bossche *et al*, 1978). However MMI and MI did not affect the chemotaxis, chemokinesis or random movement of human PMN as assayed by the method of Nelson *et al* (1975), and it was concluded that these imidazoles, like the less substituted ones, metrinidazole and tinidazole do not affect membrane permeability and thus do not affect chemotaxis.

Interaction of a leuko-attractant with PMNs *in vivo* causes increased HMPS activity, with resultant H_2O_2 and O_2^- production

as well as degranulation with the release of myeloperoxidase (Badwey and Karnovsky, 1980; Klebanoff, 1968). It has been shown that release of these compounds into the extra-cellular environment can cause auto-oxidation of the PMN, and thus decreased motility (Anderson, 1981a,b; Theron et al 1981; Jones and Anderson, 1983). The MPO-H_2O_2-halide system has been shown to cause oxidation of, for example, sulphydryl groups to sulphenic acids (Thomas and Aune, 1977; 1978). Jones and Anderson (1983) using horseradish peroxidase (HRPO), H_2O_2 and NaI to stimulate PMN in vitro, showed that ascorbate and cysteine protected the PMN from iodination by HRPO/H_2O_2/NaI and thus prevented inhibition of motility. Massive uncontrolled oxidation has been correlated with a rapid decrease in directional motility of PMN, thus the PMN is no longer able to "sense" the leuko-attractant gradient. Jones and Anderson (1983) suggest that these agents' protective effect results from their ability to protect PMN sulphydryl groups from oxidation by HRPO. Therefore these agents protect the PMN -SH groups by acting as reducing agents. The necessity of maintaining the PMN in a reduced state for purposes of motility and viability is well documented (Absolom and Oss, 1979; Bank et al, 1980).

MMI could act in a similar way to ascorbate and cysteine, as a reducing agent, to protect the PMN against auto-oxidation and thus loss of chemotactic activity.

Instead of exerting no affect at all, MMI and its metabolites may in fact be acting to protect the PMN primary function, allowing the first step towards elimination of micro-organisms to procede unhindered.

CHAPTER 3

THE EFFECT OF MMI AND ITS PUTATIVE METABOLITES ON THE PHAGOCYTOSIS OF A NUMBER OF SPECIES OF MICRO-ORGANISMS BY HUMAN PMN.

3.0 Introduction.

PMN are easily damaged by impurities or rough handling; excessive clumping is a sign of such damage, therefore several methods for their isolation were investigated to obtain cells as undamaged as possible.

It was important to assess to what extent if any MMI or any of its putative metabolites affected the phagocytosis of bacteria or yeasts, as impaired phagocytosis can result in the proliferation of infections. Clinical isolates obtained from Glasgow Dental Hospital (GDH) and Glasgow Royal Infirmary (GRI) microbiology departments were used along with a reference strain of Candida albicans as phagocytic stimuli.

3.1 Materials And Methods.

1. Isolation Of PMN

Venous blood was withdrawn, at the same time each morning, between 9 and 10 a.m. from healthy, human, male volunteers, aged between 20 and 30 years. The blood was immediately mixed with preservative-free heparin in phosphate buffered

saline (PBS). The blood was mixed with Plasmagel (Laboratoire Roger Bellon) in a ratio of 1 part Plasmagel to 2 parts blood, and the red blood cells were left to sediment at 37°C for 30 minutes. The top layer containing leukocytes and plasma was withdrawn and layered on to Ficoll-Hypaque (Pharmacia). The mixture of leukocytes was separated by centrifugation at 250 *g* for 30 minutes. The top layers containing plasma, Ficoll-Hypaque and mononuclear cells were removed and the pellet containing granulocytes was resuspended in Hanks basic salt solution (HBSS) (Gibco). Any remaining erythrocytes were hypotonically lysed and the PMN were washed and resuspended in HBSS to give a concentration of 10^7 cells per ml.

The cell preparations were stained with Wright's stain (Gurr), and contained on average 95% PMN. Viability was checked by trypan blue exclusion.

2. One Step PMN Separation From Whole Blood

Materials

A 9% (w/v) solution of Ficoll 400 (Pharmacia) made up in distilled water.

Ficoll-Hypaque (Histopaque, Pharmacia).

50% Conray solution made by diluting Conray 480 (May and Baker Ltd) with an equal volume of distilled water.

Method

A dense Ficoll-Hypaque solution was made by adding 10 parts of the 50% solution to 20 parts of 9% Ficoll. The dense solution was aliquotted in two ml amounts into test tubes. Two ml of Histopaque was layered carefully on to the dense solution in the test-tubes. On to this was layered 3-4 ml of whole blood pre-diluted with medium to 4/5 of original. The tubes were spun at 200 g for 20 minutes at room temperature.

3. One Step Separation Of PMN From Blood (Ferrante and Thong, 1978)

Materials

85% solution of Hypaque (Pharmacia)

9% solution of Ficoll 400 (Pharmacia)

Method

20 ml of Hypaque was mixed with 90 ml of Ficoll to give a density of 1.095 g/ml. The Ficoll-Hypaque was aliquotted into test tubes in three ml amounts. Five ml of whole blood was gently layered on to the Ficoll-Hypaque and the tubes were spun at 200 g for 20-30 minutes. The cell pellet was washed three times in medium.

4. A Single Step Centrifugation Method For Separation Of Granulocytes And Mononuclear Cells From Blood Using A Discontinuous Gradient Of Percoll (Giudicelli et al, 1982)

Materials

Stock isotonic solution (SIS) was made by diluting 9 volumes of Percoll with 1 volume of 1.5 M NaCl

Solution A with a density of 1.078 g/ml was made by diluting SIS to 62% (w/v) in calcium and magnesium-free Hanks basic salt solution (Gibco).

Solution B with a density of 1.094 g/ml was made by diluting SIS to 76% (w/v) in Ca and Mg-free HBSS.

Method

Three ml of solution A was aliquotted into tubes, on to this was layered three ml of solution B. Whole blood diluted twice in HBSS was carefully layered on to solutions A and B. The tubes were centrifuged at 600 g for 20 minutes at 15°C. The cells from the bands were washed twice with PBS.

5. An Improved Method For Rapid Layering Of Ficoll-Hypaque Double Density Gradients Suitable For Granulocyte Separation (Madyastha et al, 1982)

Materials

1% methylcellulose-15 (Sigma).

9% Ficoll-400 (Pharmacia).

50% and 33.9% Hypaque (Pharmacia).

Method

Solution A was made by mixing 10 parts of 50% Hypaque with 20 parts of 9% Ficoll, to give a specific gravity of 1.12.

Solution B was made by mixing 10 parts of 33.9% Hypaque with 24 parts of 9% Ficoll, to give a specific gravity of 1.077.

Both solutions were stored at 4°C in suitable aliquots (1.5 ml). Tubes containing solution A were covered with parafilm and refrigerated. Blood was mixed with 1%

methylcellulose-15 in a ratio of 4:1 and centrifuged at 30 g for 7-10 minutes at room temperature. The leukocyte-rich plasma was withdrawn and used for granulocyte separation. The Ficoll-Hypaque gradients were prepared by using 20-gauge, one inch needles attached to disposable syringes at a 45° angle on top of the test tubes to gently layer solution B on to solution A already in the test tubes. This results in a sharper interface.

Maintenance Of Cultures

The organisms Staphylococcus aureus (NCTC 6571, Oxford strain), Streptococcus pyogenes (clinical isolate GDH 1554), Escherishia coli (clinical isolate GRI 0207), Lactobacillus casei (NCTC 6375) and Candida albicans (MRL 3153) were freeze dried and a fresh ampoule of each was opened every 3 to 4 months, inbetween times the micro-organisms were maintained on nutrient agar slants at 4°C.

The micro-organisms were sub-cultured from the agar slants on to horse blood agar plates and incubated overnight at 37°C. An innoculum from these plates was used to seed 2.5% nutrient broths in 25 ml aliquots in glass universals, these were also incubated at 37°C overnight. The Candida was grown in Sabouraud broth (Difco) at 30°C for five days, to prevent germ tube formation when exposed to serum.

The cultures were centrifuged at 1500 g and the pellet was washed three times in phosphate buffered saline (PBS). The micro-organisms were counted using a Neubauer counting chamber, and the concentration adjusted to give 10^7 cells per ml of medium. (Hanks basic salt solution plus 0.1% gelatin (HBSSG)).

Opsonisation

The micro-organisms were pre-opsonised in normal human serum. The optimum concentration of serum required to give maximum phagocytosis was determined. The micro-organisms were suspended in HBSSG, plus serum giving final concentrations of 10, 20 and 40 or 50%. The cultures plus serum were incubated at 37°C with shaking for 15 to 30 minutes and the micro-organisms sedimented by centrifugation at 1500 g for 10 minutes and resuspended in HBSSG.

Phagocytosis Assay

Equal volumes of PMN and micro-organism, both at concentrations of 10^7 per ml were mixed together and incubated at 37°C with end-over-end rotation to ensure efficient mixing. Aliquots of 0.5 ml were removed after 0, 30, 60 and 120 minutes and added to 1.5 ml of ice-cold HBSSG in plastic test tubes. The tubes were centrifuged at 110 g for 4 minutes. The supernatant was withdrawn and serially diluted, aliquots of

the dilutions were spread onto blood agar plates, and incubated overnight at 37°C. The colonies were counted and averaged over several dilutions to give a measure of the number of organisms remaining un-phagocytosed in the PMN-micro-organism mixture. Controls were included to monitor the effect of serum on the viability of the bacteria and yeast, and separately to see the effect of shaking on the phagocytic mixture. The mixture was also examined microscopically.

Determination Of The Effect Of MMI, 3-MTH, MH And 1-MI On The Phagocytosis Of Micro-organisms

The various compounds were added in concentrations of between 10^{-5} M and 10^{-3} M dissolved in HBSSG to the micro-organism/PMN mixture and the assay carried out as above. Controls were included to assess the effect of the compounds on the viability of the micro-organisms. The PMN were examined microscopically and their viability, after treatment was assessed by trypan blue exclusion.

3.2 Results.

Comparison Of Methods Used To Isolate PMN

Five different methods for the isolation of PMN from whole blood were compared. Of the five methods, method 1 proved

to be the most reliable. Methods 2 and 3 were not readily reproducible, the PMN band of cells tended to be rather 'fuzzy', and only after repeated attempts using different spin speeds did a tight band of PMN appear after centrifugation. These methods were discarded as being too inconsistant. Method 4 proved to be a relatively expensive way of separating cells as it used Percoll density gradients to separate the PMN from other blood cells. Method 5 was reproducible and gave tight bands of cells, however the Ficoll-Hypaque solutions rapidly deteriorated, even whilst refrigerated at $4^{o}C$, and it was very time-consuming to have to make fresh solutions daily. Method 1 was the method of choice, despite being longer to carry out and resulting in a mixture of granulocytes and erythrocytes. A number of ways to remove the erythrocytes were tried, to find a method that caused a minimum of damage to the PMN. Hypotonic saline was chosen in preference to distilled water and dilute ammonium chloride as the former caused much cell damage and the latter appeared to cause aggregation of the PMN.

Opsonisation

Opsonisation, with 10% normal human serum incorporated into the phagocytic mixture of micro-organisms and PMN was initially used, but the serum in the mixture appeared to provide essential growth substances for the organisms and as a result of its

addition, the micro-organisms grew within the incubation times used, eventually overwhelming the PMN. This method of opsonisation was therefore discontinued.

Pre-opsonisation of micro-organisms in normal human serum overcame the problem of the organisms reproducing whilst in the phagocytic mixture. Each of the micro-organisms were pre-opsonised in 10, 20 and 40 or 50% serum, to gauge the optimal concentration resulting in optimum phagocytosis of the various micro-organisms. For S.aureus, S.pyogenes, L.casei and C.albicans the optimal serum concentration of those tried was 10%, concentrations of serum greater than this did not result in a significantly greater amount of phagocytosis. E.coli differed from the other organisms in requiring 40% serum to give optimal phagocytosis, however even using pre-opsonisation, this concentration of serum allowed the organisms to reproduce and a correction factor to take account of this had to be calculated. However the maximum incubation time for this organism had to be reduced from 120 minutes to 60 minutes as after this length of time the E.coli had over-grown the phagocytic mixture.

The Effect Of MMI On The Phagocytosis Of S.aureus, S.pyogenes, E.coli, L.casei And C.albicans

The effect of 10^{-3} M MMI on the phagocytosis of a number of micro-organisms was assessed as a preliminary study. The

Table 3

Table of mean values ± standard deviation for determinations of the percentage organisms phagocytosed over periods of time. Students' t-test applied to the results. The figures represent the means of 3 separate determinations performed in triplicate. MMI was used at a concentration of 10^{-3} M.

Using t-test, phagocytosis was found to be unaffected by MMI at a concentration of 10^{-3} M.

*Opsonised with pooled, frozen, human serum

Table 3

Effect Of 10^{-3} M MMI On The Phagocytosis Of Various Organisms*

Percentage Reduction In Numbers Of Organisms

	30 minutes incubation $\bar{x}\pm s$	t-value	60 minutes incubation $\bar{x}\pm s$	t-value	120 minutes incubation $\bar{x}\pm s$	t-value
S.aureus						
Control	70.46±1.95	0.477	79.20±3.07	1.143	88.23±1.16	0.682
MMI	71.59±3.61	NS	82.43±3.81	NS	88.87±1.14	NS
S.pyogenes						
Control	85.26±0.93	0.712	94.98±7.56	0.031	96.00±4.38	0.123
MMI	83.62±3.88	NS	95.15±5.56	NS	96.38±3.09	NS
C.albicans						
Control	95.03±1.65	0.033	98.43±0.79	0.759	99.71±0.15	2.479
MMI	94.97±2.65	NS	98.87±0.62	NS	99.23±0.30	NS
E.coli						
Control	60.86±1.92	0.900	87.33±3.02	0.294		
MMI	49.89±17.13	NS	87.91±1.59	NS		
L.casei						
Control	91.84±4.97	0.004	97.94±1.44	0.086	98.77±0.77	0.321
MMI	91.82±5.91	NS	98.03±1.11	NS	98.95±0.59	NS

Table 4

Table of mean ± standard deviation of the percentage reduction in numbers of S.aureus over 30 and 60 minutes incubation. The figures represent the mean of 3 separate determinations performed in triplicate. Correlation coefficient statistics were applied to the results. No significant dose-related effect was found due to any of the compounds.

*Opsonised in fresh, normal human serum

Table 4

Effect Of MTH, MH And MI On The Phagocytosis Of S.aureus*

Percentage Reduction In Numbers Of Organisms

Log conc drug	30 minutes incubation $\bar{x}\pm s$	r value	60 minutes incubation $\bar{x}\pm s$	r value
MTH				
0	89.90±8.92	0.029	90.61±7.92	-0.035
-3	90.63±7.99	NS	90.68±8.06	NS
-4	90.22±10.16		91.66±7.52	
-5	90.47±10.55		91.39±9.06	
MH				
0	99.44±0.40	-0.153	99.40±0.62	-0.006
-3	99.12±0.62	NS	99.42±0.52	NS
-4	99.38±0.37		99.48±0.40	
-5	99.04±0.77		99.41±0.56	
MI				
0	96.78±0.41	0.079	99.25±0.34	0.442
-3	97.07±1.50	NS	99.49±0.18	NS
-4	97.01±2.24		99.23±0.34	
-5	96.75±1.88		99.26±0.30	

Table 5

Table of mean ± standard deviation of the percentage reduction in numbers of S.pyogenes over 30 and 60 minutes incubation. The figures represent the mean of 3 separate determinations performed in triplicate. Correlation coefficient statistics were applied to the results. No significant dose-related effect was found due to any of the compounds.

*Opsonised in fresh, normal human serum

Table 5

Effect Of MTH, MH And MI On The Phagocytosis Of S.pyogenes*

Percentage Reduction In Numbers Of Organisms

Log conc drug	30 minutes incubation $\bar{x}\pm s$	r value	60 minutes incubation $\bar{x}\pm s$	r value
MTH				
0	96.89±1.25	0.158	98.44±0.26	-0.260
-3	97.63±1.03	NS	98.25±0.84	NS
-4	97.67±0.38		97.85±0.98	
-5	97.67±0.59		98.37±0.66	
MH				
0	98.05±1.20	-0.173	98.55±1.34	0.021
-3	97.58±0.03	NS	98.70±1.27	NS
-4	97.58±0.82		99.05±1.06	
-5	97.73±0.61		98.52±1.01	
MI				
0	96.98±0.73	-0.070	98.52±1.86	0.934
-3	97.29±0.30	NS	99.79±0.01	NS
-4	97.71±1.09		98.70±1.56	
-5	97.58±0.03		98.89±1.26	

results are shown in Table 3. (Students' t-test statistic was applied to the results, and it was found that MMI did not significantly affect the phagocytosis of any of the micro-organisms.)

The Effect Of MTH, MH And MI On The Phagocytosis Of S.aureus And S.pyogenes

S.aureus and S.pyogenes were chosen as representatives of H_2O_2- and non-H_2O_2- producing organisms to investigate the influence of MTH, MH and MI, some of the putative metabolites of MMI, on the phagocytosis of bacteria. The results are shown in Tables 4 & 5. Correlation coefficient statistics were applied to the results to see if there was a dose-related effect due to the drugs over a concentration range 10^{-5} to 10^{-3} M. The analysis indicated that MTH, MH and MI do not affect phagocytosis of either organism in a dose-related or any other manner, over 60 minutes incubation.

Viability Of Organisms In The Presence Of MMI, MTH, MH And MI

The compounds were found to have no effect on the viability or growth of any of the organisms over a 60 minute incubation time.

3.3 Discussion.

There are a number of methods which have been described in

the study of phagocytosis by PMN. These include assays measuring the removal of a test particle from the fluid medium surrounding the cells, quantitative assays counting the number of particles taken up by a cell, and indirectly by assays measuring metabolic change during the phagocytic process such as measurement of chemiluminescence and flow cytometry, the former in which phagocytosis is estimated by measurement of luminescence using a liquid scintillation spectrometer or luminometer, the latter a fluorescence activated cell sorter.

The method used in this study involves measurement of the removal of micro-organisms from the medium surrounding the PMN. The technique is simple to perform, and has an immediate relevance to the problem of PMN-micro-organism interaction: there is scope for examining the effects of drugs or inhibitors on the system. However it is limited by its laboriousness, and long incubation times that foster cell-cell, particle-particle and cell-particle clumping, and does not differentiate between non-specific attachment of particles to cell surfaces from actual internalisation of the particles by the cells. The alternatives such as direct counting by light microscopy or electron microscopy or measurement of radioactivity of radio-labelled phagocytosed particles also have these drawbacks, but in addition are more time consuming and expensive.

The need for opsonisation of micro-organisms for ingestion to occur has been extensively studied since Wright and Douglas (1903) first described the opsonic effect of serum. They demonstrated that the action of the serum was on the bacteria rather than on the phagocytic cell.

In contrast Wood, Smith and Watson (1946) reported that phagocytosis and killing can occur in the absence of opsonins when the bacteria are trapped by the PMN against a surface. This mechanism of phagocytosis is termed 'surface phagocytosis'. More recently it has been shown that the surface charge and hydrophobicity of the bacteria are important factors that determine whether micro-organisms need opsonins or not for uptake by the PMN (Van Oss and Gilman, 1972a,b; Stendahl and Edebo, 1972; Stendahl et al, 1979).

Uptake of opsonised bacteria and triggering of PMN activation proceeds via a receptor on the PMN membrane for the Fc fragment of the Ig molecule and via a receptor for the C3b molecule. Under conditions where PMN are incubated with bacteria with constant shaking, virtually no uptake is measured when opsonins are absent (Verhoef et al, 1977; Verbrugh et al, 1979). Thus the need for opsonisation during determination of phagocytosis has been shown. Peterson et al (1977) investigating the phagocytosis of different strains of S.aureus demonstrated that increasing the

concentration of serum from 10% to 100% did not change the opsonic activity of heat inactivated serum. Vandenbroucke-Graulls et al (1984) showed that uptake of S.aureus proceeds at a slower rate, but induced respiratory burst activity to the same extent as opsonised bacteria. In the present study opsonisation using serum concentrations from 10% to 50% did not significantly alter the amount of phagocytosis of S.aureus, S.pyogenes, L.casei or C.albicans. E.coli was exceptional in requiring 40% serum for optimum opsonisation. In the assays determining the effect of MMI on the phagocytosis of the various micro-organisms, frozen, pooled, human serum was used for opsonisation, whereas in the assays determining the effects of MTH, MH and MI on the phagocytosis of S.aureus and S.pyogenes fresh serum was used. The fresh serum appears to result in more phagocytosis of micro-organisms than does the frozen serum, where the complement components will have perished. This perhaps demonstrates the need for opsonins from both the classical and alternative complement pathways.

Phagocytosis of micro-organisms by PMN is accompanied by changes in the cell membrane and cytoplasm of the PMN, and by an increase in oxygen consumption and metabolic activity (Cohn and Hirsch, 1960; Ingraham et al, 1981; Lane et al, 1981; Root et al, 1975; Borregaard and Herlin, 1982) although this oxygen consumption is not required for phagocytosis (Koch, 1978). Thus phagocytosis is the initiating event for

the respiratory burst during which a number of toxic oxygen species are produced. Lam and Lindsay (1979) showed that phagocytosing PMN accumulate ^{14}C-MMI and ^{14}C-PTU. They found that PTU accumulation was accompanied by an increase in H_2O_2 availability in phagocytosing PMN, and suggested that PTU accumulation was related to its oxidation, presumably brought about by the increased production of reactive oxygen species by the stimulated PMN. It seems likely that the same would apply to MMI. MMI is known to be metabolised by the PMN, and MMI has been identified as a metabolite (Skellern, in press). Thus it was important to assess whether MMI or any of the putative metabolites of the drug would exert any effect on phagocytosis, especially as it initiates the respiratory burst.

Micro-organisms are phagocytosed at different rates. The differences observed can be explained by the possession of the organisms of anti-phagocytic properties. S.aureus has protein A as a cell wall component of most strains. It binds to the Fc portion of human IgG subclasses IgG1, IgG2 and IgG4 (Kronvall and Williams, 1969). Protein A is believed to be anti-phagocytic; by binding IgG molecules at their Fc portion, protein A would prevent these molecules from participating in IgG-mediated phagocytosis, which is Fc receptor-dependent. However, in the presence of complement,

the phagocytosis-inhibiting properties of protein A are dependent on the amount of IgG present. This may be why the amount of phagocytosis of S.aureus increased from 70% to 90% when fresh serum was used for opsonisation rather than frozen where most of the complement components were destroyed.

A substance functionally analogous to but immunologically distinct from protein A has been reported on group A Streptococci (Schalen et al, 1978), the S.pyogenes used in the phagocytic assays here was a group A Streptococcus, and a similar increase was found to that seen with S.aureus, when fresh serum was used for opsonisation, from 85% to 97-98%. Group A Streptococci also contain M proteins, which are cell surface antigens and major virulence determinants. Their enhancement of virulence is believed to be related to an anti-phagocytic effect (Lancefield, 1962), possibly as a result of masking cell wall constituents capable of fixing complement to the cell surface (Horwitz, 1982).

Micro-organisms have a range of strategies used to avoid phagocytosis and killing, which include pili, capsules and a host of enzymes. Thus these and other factors determine the rate and amount of phagocytosis of micro-organisms by PMN.

The chemiluminescent response of normal human PMN to a range

of pathogenic micro-organisms was investigated by Robinson et al (1984). They found that measuring the luminol-enhanced chemiluminescence response of PMN to a number of organisms gave reproducible response patterns, and correlating chemiluminescence with phagocytic and bactericidal activity, deduced that the response patterns reflected differences in cell wall and membrane characteristics of the micro-organisms being phagocytosed.

Initially, the phagocytosis of five micro-organisms was investigated, however after assessing the effect of MMI on phagocytosis and killing, the original five organisms were reduced to two, S.aureus and S.pyogenes. These were taken as being representative, both being highly pathogenic and therefore clinically relevant, the Streptococcus being an H_2O_2-producing organism and the Staphylococcus a non-producer.

Klebanoff and White (1969) found that MMI did not significantly affect the phagocytosis of Lactobacillus acidophilus by normal human PMN, and in 1972, Klebanoff and Hamon determined that the phagocytosis of S.aureus was unaffected by MMI. Ferguson et al (1984) found that the phagocytosis of opsonised zymosan by human PMN was unaffected by MMI. Lehrer (1975) investigating the fungicidal mechanisms of human monocytes found that phagocytosis of Candida albicans by human monocytes was

unaffected by MMI.

In this study, MMI and its putative metabolites did not significantly affect the phagocytosis of S.aureus, S.pyogenes, L.casei, E.coli or C.albicans.

CHAPTER 4

The Effect Of MMI And Its Putative Metabolites On The Intracellular Killing Of S.aureus, S.pyogenes, L.casei, E.coli And C.albicans

4.0 Introduction.

Intracellular killing can be investigated in many different ways. In this study several methods are used, killing is monitored directly: by a plate count method measuring the decrease in numbers of phagocytosed micro-organisms, visualised microscopically using a vital stain to differentiate between viable and nonviable micro-organisms, and indirectly using a luminometer to detect luminol-enhanced chemiluminescence from stimulated PMN.

4.1 Materials And Methods.

Preparation of PMN, micro-organisms and drugs was as previously described (3.1).

Killing Assay (1)

Equal volumes of PMN and micro-organism at concentrations of 10^7/ml were incubated together with end-over-end mixing at 37°C for up to two hours. Samples were removed at various intervals and diluted one in five in ice cold HBSSG. The tubes were centrifuged at 800 *g* for four minutes. The supernatant was removed, and further serial dilutions were performed and

aliquots plated out, to give a measure of phagocytosis. The pellet was resuspended in ice-cold distilled water plus 0.01% bovine albumin (Sigma), and pipetted gently for about 30 seconds to lyse the PMN. Serial dilutions of the lysed PMN were made and plated onto horse blood agar plates and incubated overnight at 37°C.

Killing Assay (2)

Equal volumes of PMN and micro-organism at concentrations of 10^7/ml were incubated together with end-over-end rotation at 37°C for 15 minutes (to allow phagocytosis to take place). The PMN-micro-organism suspension was centrifuged at 110 g for four minutes, to sediment the PMN containing micro-organisms. The supernatant containing un-phagocytosed organisms was discarded, and the pellet was washed twice with HBSSG to remove any organisms left un-phagocytosed. After washing, the pellet was resuspended in fresh HBSSG and incubated with end-over-end shaking at 37°C, samples were removed at various time intervals, diluted in ice-cold HBSSG and centrifuged at 800 g for four minutes. The supernatant was discarded and one ml of ice-cold distilled water plus 0.01% bovine albumin was added and the suspension was pipetted for 30 seconds to disrupt the PMN. Serial dilutions were made and plated as before.

Fluorochrome Microassay (Assessment of blood leukocyte microbial killing)

Method modified from Pantazis and Knicker, 1979.

PMN preparation was as before, except that the cells were suspended in PBS at a concentration of 10^6PMN/ml. Micro-organisms' preparation and opsonisation was as before except that the cells were suspended in McCoy's medium 199 (Gibco) to give a concentration of 8 X 10^6/ml.

Fluorochrome Microassay

Two hundred µl of the PMN suspension was dripped onto washed, circular 18 mm-diameter coverslips. Each of the coverslips was supported by a saline-moistened sponge, 9 mm in diameter and 5 mm high, and placed in a petri dish. The petri dishes were then covered and carefully placed in a 37°C, humidified, 5% CO_2 incubator for 60 minutes to allow the PMN to adhere to the coverslips. After 30 minutes several coverslips were withdrawn and stained with filtered Wright's stain. After 60 minutes the coverslips were washed gently in HBSS warmed at 37°C and carefully returned to the petri dishes. One hundred µl of the opsonised C.albicans suspension was added to each of the coverslips, which were returned to the incubator. At various time intervals a coverslip was removed from the petri dishes, washed gently in warmed HBSS and transferred monolayer side up to a petri dish containing 30 to 50 ml of a freshly-made 0.01% solution of acridine orange (Gurr) made up in 0.1 M citric acid/NaOH buffer (pH 5). The coverslips were stained for 60 seconds, then removed from the stain and excess dye removed by touching the edge of the coverslip to a dry paper towel. The coverslips were

then mounted onto glass slides and sealed with clear nail polish and examined with a UV fluorescent microscope using a halogen light source and with a x100 water immersion objective.

MMI at a concentration of 10^{-3} M was incorporated into the assay to test its effect on phagocytosis and killing of C.albicans.

Chemiluminescence Assay

Preparation of PMN, opsonised micro-organisms and drugs as before (3.1).

Preparation Of Opsonised Latex And Zymosan

Zymosan A (Sigma) was suspended in sterile, normal human serum at a concentration of 10 mg/ml. The suspension was incubated with shaking at 37°C for 30 minutes. At the end of this period the zymosan/serum mixture was centrifuged at 1800 g for ten minutes, the supernatant removed and the pellet of opsonised zymosan resuspended in Hanks basic salt solution plus gelatine, without phenol red.

The latex spherules (0.8 μ diameter, Sigma) were prepared similarly, to give a concentration of 10^8 particles/ml.

Preparation Of Luminol

Luminol (Sigma) was dissolved in dimethylsulphoxide (DMSO)

to give a final concentration of 10^{-2} M. This was kept as stock solution at -20°C. From this a working solution was made up in PBS to give a concentration of 1.2 X 10^{-4} M, this was further diluted 1 in 10 during the chemiluminescence assay. The luminol was always freshly prepared on the day of use.

Chemiluminescence Assay

Two hundred µl of PMN at a concentration of 1.0 - 5.0 X 10^{6} cells/ml in HBSS without phenol red indicator, plus 50 µl of either test compound at concentrations ranging from 10^{-3} to 10^{-7} M, or HBSS were added to glass tubes and placed in the chamber of the Packard Pico-lite Luminometer which was maintained at 37°C. Phagocytosis was initiated by injecting opsonised latex or zymosan into the glass tubes within the luminometer using a Hamilton syringe. The luminometer was programmed to measure chemiluminescence for 30 seconds every 60 seconds for up to 60 minutes.

Viability Of Micro-organisms In The Presence Of The Various Drugs

All combinations of the drugs and micro-organisms were incubated together at 37°C for 60 minutes. Samples were withdrawn at 15 minute time intervals and plated onto blood agar plates to check viability in the presence of the various compounds.

4.2 Results.

Effect Of 10^{-3} M MMI On The Intracellular Killing Of Various Micro-organisms

The intracellular killing of S.aureus, S.pyogenes, E.coli, L.casei and C.albicans by human PMN, and the effect of 10^{-3} M MMI on it was assessed by killing assay 2. The results are expressed as the percentage of phagocytosed organisms rendered non-viable after 30, 60 and 120 minutes incubation. (Table 6)

MMI appeared to stimulate intracellular killing of all five micro-organisms to greater and lesser extents over 30 minutes, however following 60 and 120 minutes, S.aureus and C.albicans showed slightly less killing in the presence of MMI and L.casei and S.pyogenes a slightly greater percentage.

Effect Of MMI, MTH, MH and MI On The Intracellular Killing Of S.aureus and S.pyogenes

The effect of the four compounds at concentrations of 10^{-3}, 10^{-4} and 10^{-5} M on the intracellular killing of S.aureus and S.pyogenes over 30 and 60 minutes incubation periods are shown in Tables 7 and 8 respectively.

The intracellular killing of S.aureus showed significant dose-related inhibition in the presence of MMI and MTH, the inhibition shown was slight but consistant. MI and MH had no apparent effect

The Effect Of MMI On The Killing Of Various Micro-organisms By Human Polymorphonuclear Leukocytes.

Percentage Reduction In Numbers Of Organisms

	30 minutes incubation	60 minutes incubation	120 minutes incubation
S.aureus			
Control	48.33	75.67	77.67
MMI	63.67	74.33	77.33
S.pyogenes			
Control	61.67	81.67	94.67
MMI	82.67	86.00	97.33
C.albicans			
Control	76.33	95.33	97.97
MMI	87.33	93.33	96.00
L.casei			
Control	37.50	90.00	98.90
MMI	78.00	96.00	99.50
E.coli			
Control	35.19		
MMI	46.30		

Table 6

Table of the mean of two separate determinations performed in duplicate, percentage reduction in numbers of organisms over time, and the effect of MMI at a concentration 10^{-3} M on intracellular killing.

on the ability of human PMN to destroy ingested micro-organisms (Figs 15&16). None of the compounds tested had any significant effect on the intracellular destruction of S.pyogenes.

Assessment Of Intracellular Killing Of C.albicans By Fluorochrome Microassay

Viable C.albicans fluoresced either green or orange in colour under a U.V. microscope. PMN stained green, ingested Candida stained orange and non-viable C.albicans stained red. After 60 minutes incubation, it was found that on average 55.2% of ingested C.albicans were non-viable, however it was difficult to differentiate clearly between viable and non-viable cells, particularly as some tended to stain brownish ie. between orange and red.

The correlation between methylene blue staining (vital stain) and acridine orange as a vital stain in extracellular organisms was 100%, however it was not possible to extend this assay to intracellular organisms.

Influence Of MMI, MTH, MH And MI On The Generation Of Chemiluminescence By PMN Stimulated By Opsonised Zymosan Or Latex Particles

Opsonised zymosan tended to form aggregates, and when used to stimulate PMN, the chemiluminescence generated reflected the

Table 7

Table of mean ± standard deviation of the percentage reduction in numbers of S.aureus over 30 and 60 minutes incubation. The figures represent the mean of 9 separate determinations performed in triplicate. Correlation coefficient statistics were applied to the results, the r values are shown, along with the corresponding P value.

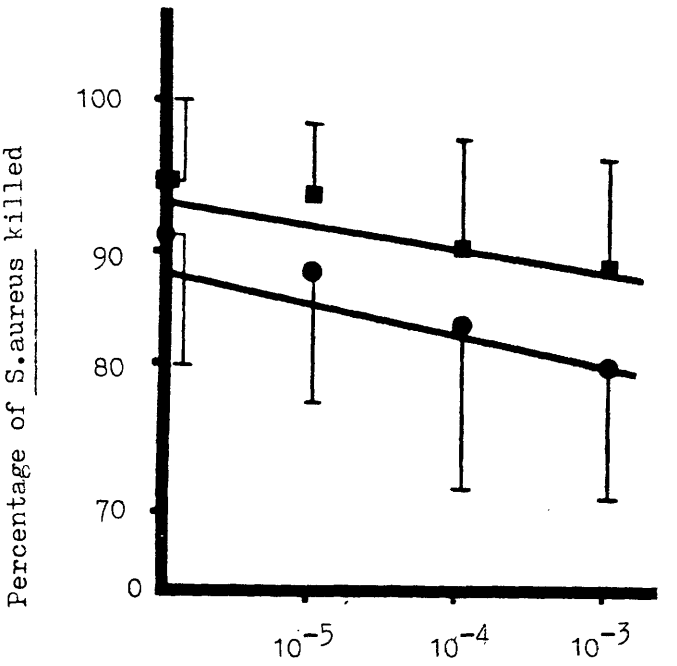
Table 7

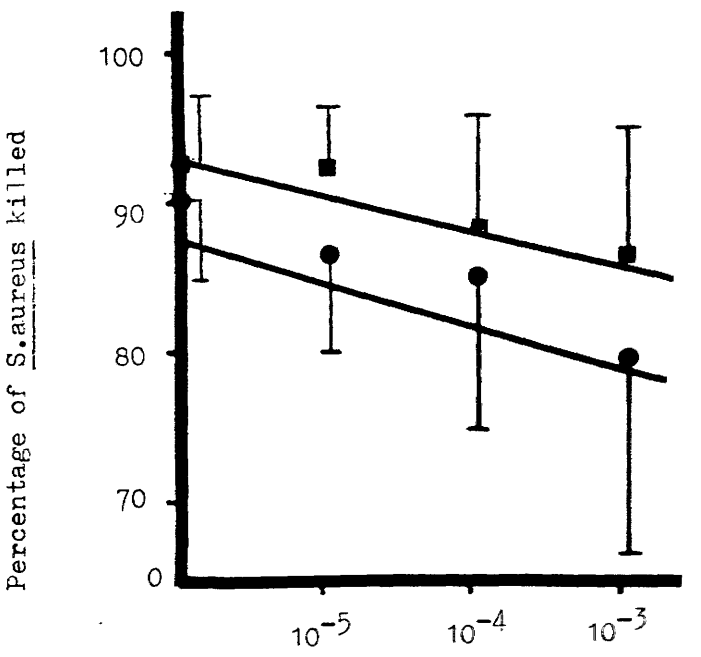
Effect Of MTH, MH, MI And MMI On The Killing Of S.aureus.

	Percentage reduction in numbers of organisms.			
Log conc drug	30 minutes incubation	r value	60 minutes incubation	r value
Methylthiohydantoin	x±s		x±s	
0	90.95±5.26	-0.402	93.08±4.22	-0.347
-5	87.16±6.57		92.51±3.95	
-4	85.32±10.27	P=0.02-0.1	88.58±7.66	P=0.05-0.1
-3	79.28±12.84		86.64±8.25	
Methylhydantoin				
0	87.51±7.90	0.048	89.84±6.10	0.110
-5	87.59±8.46		91.54±5.64	
-4	89.25±6.72	N.S.	89.20±7.65	N.S.
-3	88.76±6.58		91.82±5.64	
Methylimidazole				
0	87.51±6.25	-0.177	90.70±4.87	-0.193
-5	87.10±7.76		91.33±3.73	
-4	86.20±8.06	N.S.	91.58±4.49	N.S.
-3	83.74±10.70		88.87±7.34	
Methimazole				
0	88.66±8.57	-0.291	92.80±4.92	-0.311
-5	86.62±9.05		91.53±4.91	
-4	83.05±11.28	P=0.05-0.1	88.40±6.86	P=0.05-0.1
-3	80.16±8.87		86.78±7.58	

Figure 15

Influence of MMI and MTH at concentrations ranging from 10^{-3} to 10^{-5} M on the intracellular killing of S.aureus after 30 and 60 minutes incubation





FIGURES 15 and 16

LEGEND

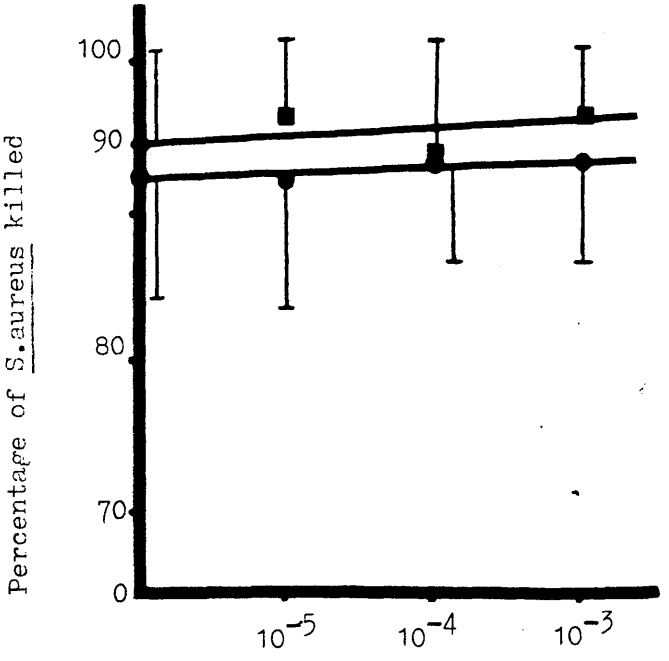
● - ● 30 minutes incubation

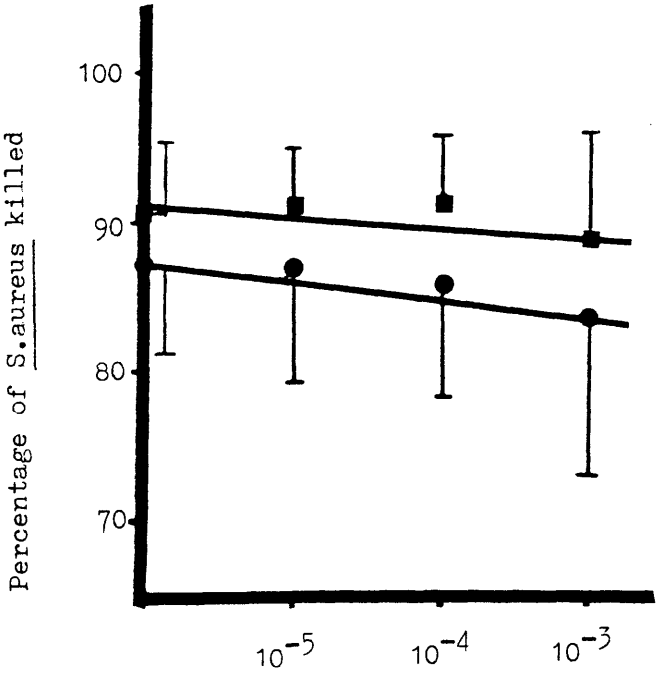
■ - ■ 60 minutes incubation

Each point represents the mean ± standard deviation of nine determinations.

Figure 16

Influence of MI and MH on the intracellular killing of S.aureus after 30 and 60 minutes of incubation.





Effect Of MTH, ME, MI And MMI On The Killing Of S.pyogenes.

Log drug concentration	Percentage reduction in numbers of organisms. 30 minutes incubation	r value	60 minutes incubation	r value
Methylthiohydantoin	x±s		x±s	
0	92.08±6.66	0.018	95.83±3.50	0.186
-5	92.51±6.22		95.70±3.47	
-4	92.92±6.11	N.S.	94.92±3.74	N.S.
-3	92.66±5.65		96.78±2.62	
Methylhydantoin				
0	98.47±1.74	0.161	99.51±0.28	0.149
-5	98.95±0.67		99.42±0.20	
-4	98.58±1.21	N.S.	98.22±2.33	N.S.
-3	99.04±0.54		99.55±0.30	
Methylimidazole				
0	96.56±5.13	0.251	98.29±1.87	0.179
-5	94.85±6.58		97.93±2.52	
-4	96.76±4.82	N.S.	97.51±1.99	N.S.
-3	95.95±5.99		98.55±1.03	
Methimazole				
0	97.88±1.62		99.03±0.10	
-5	98.21±1.29	N.S.	99.24±0.88	N.S.
-4	97.99±1.18		99.21±0.96	
-3	97.59±0.97		99.06±0.98	

Table 8

Table of the mean ± standard deviation of the percentage reduction in numbers of S.pyogenes over 30 and 60 minutes incubation. The figures represent the mean of 3 separate determinations performed in triplicate. Correlation coefficient statistics were applied to the results, the r values are shown, however no significant dose-related effect was found.

N.S. = not significant

Figure 17

Legend

○ - ○ 5 Minutes incubation

● - ● 15 Minutes incubation

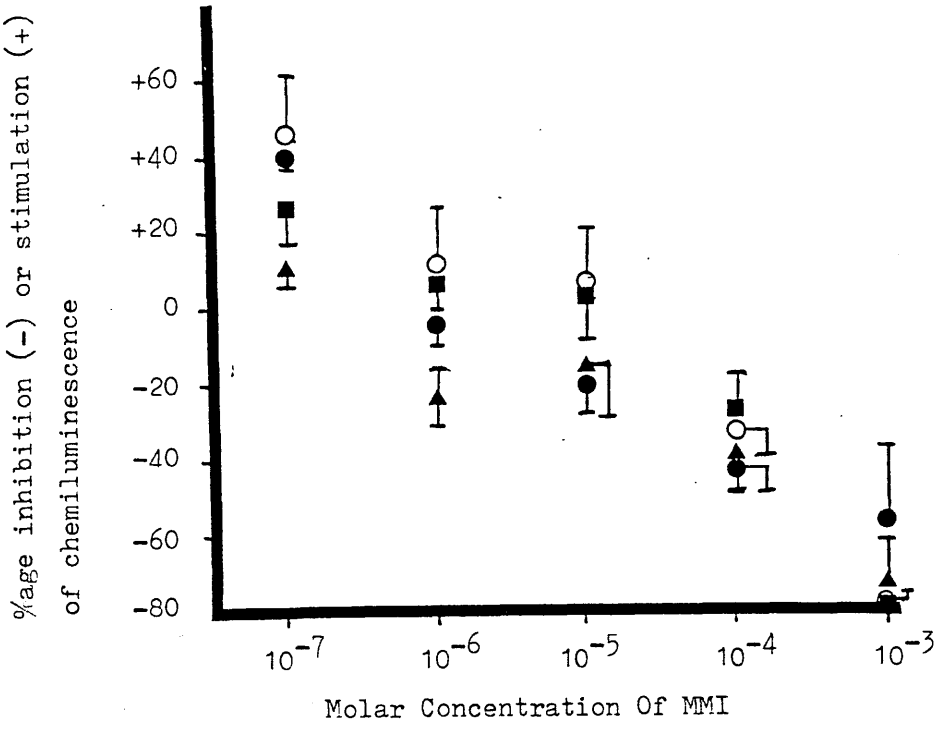
▲ - ▲ 30 Minutes incubation

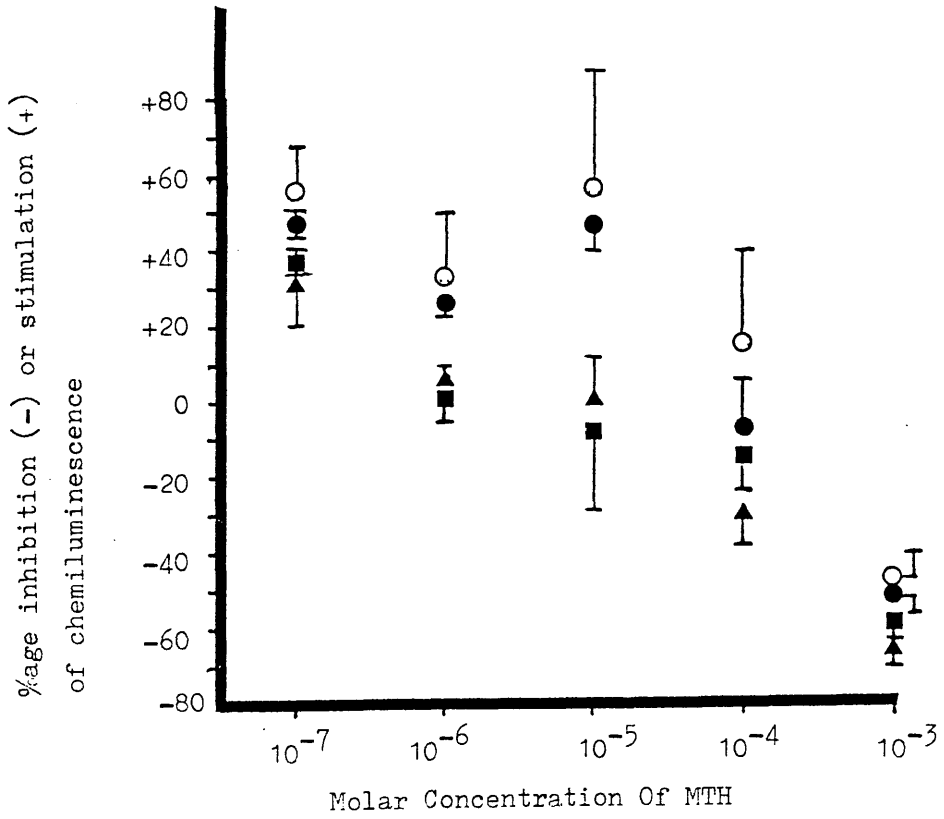
■ - ■ 60 Minutes incubation

The values shown represent the mean ± standard error of the mean of seven separate determinations. Correlation coefficient statistics were applied to the results and MMI and MTH at concentrations between 10^{-3} and 10^{-5} were found to significantly inhibit chemiluminescence in a dose-related manner. P values of 0.001 were obtained for each incubation time.

Figure 17

Chemiluminescence response of MMI- and MTH-treated latex-stimulated PMN expressed as percentage inhibition or stimulation over control levels, measured after 5, 15, 30 and 60 minutes incubation





ingestion of clumps by displaying erratic counts. Opsonised latex was therefore chosen for the study of the effect of MMI, MTH, MH and MI on PMN chemiluminescence, as this problem did not occur whilst using it as phagocytic stimulus. None of the compounds were found to alter background chemiluminescence generation. MMI and MTH at concentrations between 10^{-3} and 10^{-5} M produced dose-related inhibition of luminol-enhanced chemiluminescence between 5 and 60 minutes incubation. MH and MI did not significantly affect chemiluminescence generated over 60 minutes.

However, using untreated, latex-stimulated PMN as a reference, the inhibition decreased over time, thus 10^{-5} M MMI produces 30% inhibition after 5 minutes, but stimulates chemiluminescence production by 20% after 60 minutes. With MTH at the same concentration chemiluminescence is stimulated by 40% after 5 minutes and inhibited by 20% after 60 minutes.

In addition, the chemiluminescence of 3% H_2O_2 was inhibited and/or stimulated by MMI and MTH at concentrations between 10^{-3} and 10^{-7} M, however no reproducible results could be obtained.

4.3 Discussion.

The techniques used to monitor the killing capacity of PMN are limited, due to the fragile nature of the cells, their

activity, once isolated, diminishes with time. They have a lifetime of about six to eight hours. Direct methods of measurement tend to be laborious, therefore several methods were investigated in this study; reduction in viable numbers as seen by serial dilution and plate counts of lysed PMN or whole PMN plus micro-organisms, fluorescence microscopy differentiating viable and non-viable intracellular micro-organisms and indirectly by measuring generation of reactive oxygen species in response to stimulation by opsonised latex.

The blood fluorochrome microassay is based on the ability of acridine orange to intercalate with nucleic acids and exhibit metachromasia ie. the colour of the dye changes, towards the red, when bound to certain cellular components. Depending on the concentration of DNA and RNA in the cell, it fluoresces orange and green. When the cell becomes non-viable it fluoresces red or brown (half red, half orange). The precise mechanism behind this is unknown. The assay was extremely difficult to evaluate using bacteria due to their size, therefore the killing of C.albicans was investigated, however as the cells were not fixed, even at room temperature further inter action between the PMN and the yeast was possible. The assay was therefore felt to be unsuitable for the investigation of the effects of the various compounds on intracellular killing due to the time necessary for counting significant numbers of organisms within PMN, as well as the difficulties involved in enumerating bacteria. The assay also did not allow for shaking within the system, therefore the plate method for

assessing killing was used. This assay was more suitable, as counts of organisms did not have to be taken immediately, instead the number of micro-organisms surviving intracellularly (Method 2) or total surviving micro-organisms (Method 1) after various intervals of time were enumerated by plating diluted samples on to agar plates and incubating them overnight, one viable cell being taken as a single colony-forming unit. Once it was established that MMI, MTH, MH and MI did not alter phagocytosis or the viability of the micro-organisms, method 1 was used exclusively.

A preliminary study of the effect of 10^{-3} M MMI on the intracellular killing of micro-organisms showed that MMI stimulates killing over 30 minutes incubation, this stimulation however was not evident after 60 and 120 minutes. S.pyogenes and L.casei were rendered non-viable in the presence of MMI at rates considerably greater than those seen with S.aureus, E.coli and C.albicans. S.pyogenes and L.casei are H_2O_2-producing micro-organisms, the others are not. One representative was taken from each group for further investigation. The effect of MMI, MTH, MH and MI, at concentrations between 10^{-3} and 10^{-5} M, on the ability of human PMN to kill S.aureus and S.pyogenes was determined. MMI and MTH caused inhibition of killing of S.aureus in a dose-related manner, 10^{-3} M being responsible for approximately a 10% decrease in killing by MMI and MTH, MI was less inhibitory and MH showed no effect. The killing of S.pyogenes, in contrast, was completely unaffected by any of the compounds.

Chronic granulomatous disease is a syndrome in which the patients are unusually susceptible to infection. Their PMN are less efficient than normal in the killing of certain micro-organisms, due to the complete absence of activity of the microbicidal oxidase system (Holmes et al, 1967). Their PMN phagocytose the micro-organisms normally, but organisms such as S.aureus, E.coli and several other enteric bacilli are not killed normally. In contrast, S.pyogenes and S.faecalis are killed readily and do not play a role in the recurrent infections of these patients (Kaplan et al, 1968). Studies have shown that these PMN do not demonstrate the normal post-phagocytic increase in H_2O_2 formation (Holmes et al, 1967). Mandell and Hook (1969) correlated susceptibility to intracellular killing with H_2O_2 production, ie. organisms producing H_2O_2 were killed normally, non-H_2O_2 producing organisms remained viable and caused infection. Klebanoff and White (1969) postulated that intraleukocytic formation of H_2O_2 by bacteria could make up for the defect in H_2O_2 generation by PMN, and thus explain the ability of CGD PMN to kill certain bacteria.

The pattern of killing of micro-organisms by MMI- or MTH-treated PMN mimics that of CGD PMN, suggesting that the mechanism of action of these compounds involves H_2O_2. Klebanoff and White (1969) found that MMI did not significantly affect the killing of Lactobacillus acidophilus by normal PMN, but consistently decreased the rate of killing of the organism by CGD PMN, thus MMI inhibits in a different way from the

H_2O_2-deficient CGD PMN. Klebanoff (1972) subsequently found MMI to have a small but significant inhibitory effect on staphylocidal activity in normal PMN. Antithyroid compounds act by blocking thyroid peroxidase (TPO). PMN contain the enzyme myeloperoxidase (MPO), agents which inhibit intracellular utilisation of H_2O_2 (eg. by inhibiting MPO) favour greater recovery. The proportion of oxygen consumed which was recovered as H_2O_2 has been reported as 3%, 20-30%, 50-70% and nearly 100% during the early phagocytic period in different studies (Zatti et al, 1968; Baehner et al, 1970; Klebanoff and Hamon, 1972; Homan-Muller et al, 1975). MMI has been shown to stimulate oxygen consumption in latex-stimulated PMN (Ch. 5.2), therefore H_2O_2 is available within the cell, however the MPO activity of MMI-treated PMN is significantly decreased (Ch. 6.2). Thus it would appear that MMI acts by inhibiting MPO utilisation of H_2O_2 to effect microbial killing. Klebanoff and Pincus (1971) suggested that the inhibition or absence of MPO results in an increased utilisation of H_2O_2 in non-MPO-mediated H_2O_2-dependent reactions, including formate oxidation, HMPS activation and the iron-H_2O_2-iodide cytotoxic system. Evidence for this came from patients with hereditary MPO deficiency, who are often asymptomatic, in vitro studies with their PMN show delayed but ultimately effective microbicidal activity (Lehrer and Cline, 1969; Klebanoff and Hamon, 1972; Klebanoff, 1980). Phagocytosis induced formate and glucose C-1 oxidation by the PMN of a patient with MPO deficiency was greater than normal (Klebanoff and Pincus, 1971). The HMPS activity of

MMI, MTH and MI-treated stimulated PMN has also been shown to be increased (Ch. 5.2).

Thus the PMN has an overkill capacity and even although part of its killing capacity has been inhibited, it has many backup systems, possibly accounting for the absence of inhibition seen in the killing of S.pyogenes. However non-H_2O_2-producing micro-organisms generally possess catalase, an enzyme which catalyses the degradation of H_2O_2 to oxygen and water, thus it would be able to resist killing to some extent by the attack of backup systems using H_2O_2. The S.aureus and E.coli used in this study possessed catalase activity.

Peroxidases such as MPO, and catalase form an enzyme substrate complex with H_2O_2; the affinity of this complex for a second molecule of H_2O_2 is low as compared to its reactivity towards a variety of other compounds. Certain electron donors are oxidised by peroxidase and H_2O_2 with the formation of highly toxic products.

MMI has been demonstrated as being capable of scavenging OH•, however the products of this reaction are likely to include the drug radical cation ($MMI^{+}\cdot$) (Taylor et al, 1984). Thus the positive effect of removing OH• may be counter-balanced by the production of a potentially damaging radical. Radicals are capable of interacting with, and subsequently damaging the entire array of macromolecules which make up a cell.

MMI is metabolised by human PMN, MI has been identified as a metabolite (personal communication from G.G. Skellern) MTH and MH are also possible metabolites. MMI and MTH cause dose-related inhibition of staphylocidal activity, but MH and MI have no effect on the killing of S.aureus. Thus a time scale wherein MMI may exert an effect on PMN function could be created. It is known that MMI can be converted to $MMI^{+\cdot}$, which presumably would act on the PMN in a different manner. Depending on the amount, rate and type of metabolite produced in the course of a bactericidal assay, after a certain amount of time, the effects seen may be the result of an entirely different compound.

The preliminary studies in which MMI appeared to stimulate killing of all five micro-organisms following 30 minutes incubation, may be as a result of MMI being acted on by the active oxygen species produced by stimulated PMN. In these assays, PMN were allowed to phagocytose the test micro-organisms for 15 minutes, following this, unphagocytosed micro-organisms were removed by differential centrifugation and washing, the MMI was then added to assess its effect on intracellular killing of phagocytosed organisms, thus the respiratory burst had already taken place, and many toxic products were available to act on the PMN, the ingested organisms and the MMI. The second set of experiments measured the total effect on reduction in bacterial count, as it had been established that MMI, MTH, MH and MI did not significantly affect phagocytosis. MMI and

its putative metabolites were therefore present in the assay immediately prior to stimulation by S.aureus or S.pyogenes, and before production of reactive compounds from the respiratory burst. This may have influenced the course of metabolism of the compounds.

The stimulatory effect of 10^{-3} M MMI on the killing of ingested micro-organisms diminished over time, after 120 minutes no significant stimulation was detected, perhaps due to the metabolism of MMI to $MMI^{+\cdot}$, MTH or MI. MTH was found to inhibit staphylocidal activity to a slightly greater degree than MMI or MI.

The oxygen intermediates produced by stimulated PMN can be measured indirectly by quantitating the light emission, or chemiluminescence, that occurs when excited electrons present in these intermediates return to ground state and release photons (Allen et al, 1972). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) amplifies the chemiluminescence response and allows the use of lower numbers of cells (Allen and Loose, 1976). Luminol is presumed to be oxidised by some reactive species of oxygen generated during the respiratory burst, although the exact reaction is not defined. The oxidation of luminol results in the production of an excited aminophthallate anion that relaxes to the ground state with the production of light (Allen and Loose, 1976).

The results obtained in this study illustrate that the technique of chemiluminescence detection is not suitable for the study of certain compounds. The nature of MMI and MTH, ie. its conversion to $MMI^{+\cdot}$ or MI, the readiness of MTH to oxidise and the ability of MMI to scavenge $OH^{\cdot}$ makes the assay very difficult to interpret as the compounds may be in a different state on addition to the test system. The compounds do not affect background chemiluminescence, but do interfere with the detection of chemiluminescence, as seen by their interaction with H_2O_2 and luminol. The effect of MMI on the detection system is probably due to the production of the MMI radical which may react with luminol, possibly MTH behaves in a similar way. As it is not possible to estimate with any accuracy the extent to which MMI and MTH are responsible for quenching or stimulating chemiluminescence, the results obtained from stimulated PMN cannot be interpretted with any confidence, and are mentioned here only briefly.

Therefore in a clinical situation, it would appear that MMI probably does not exert any deleterious effects on the response of PMN to invading micro-organisms, and unless MMI treatment was coupled with some other immunological defect, is probably not responsible for any clincial disease.

CHAPTER 5

THE EFFECT OF MMI AND ITS PUTATIVE METABOLITES ON THE OXYGEN CONSUMPTION AND HEXOSE MONOPHOSPHATE SHUNT ACTIVITY OF LATEX-STIMULATED HUMAN PMN.

5.0 Introduction.

The monitoring of both oxygen consumption and hexose monophosphate shunt (HMPS) activity of PMN (Fig 16) have been used as indirect methods of assessing the killing capacity of the PMN. These methods allow for the use of inhibitors in the systems, which can be used to identify specific effects of compounds on the PMN's capacity for phagocytosis and killing.

Phagocytosis is accompanied by a number of metabolic changes, within the PMN, refered to as the respiratory burst. These include a cyanide-insensitive increase in O_2 uptake and stimulation of the HMPS.

The oxidation of glucose via the HMPS results in the production of H_2O_2 and superoxide. This event is usually measured by the conversion of (^{14}C)-1-glucose to $^{14}CO_2$ in the presence of stimulated PMN.

The increase in O_2 consumption occurring during the respiratory burst can be measured using an oxygen electrode, which detects the percentage level of O_2 saturation of a solution. In

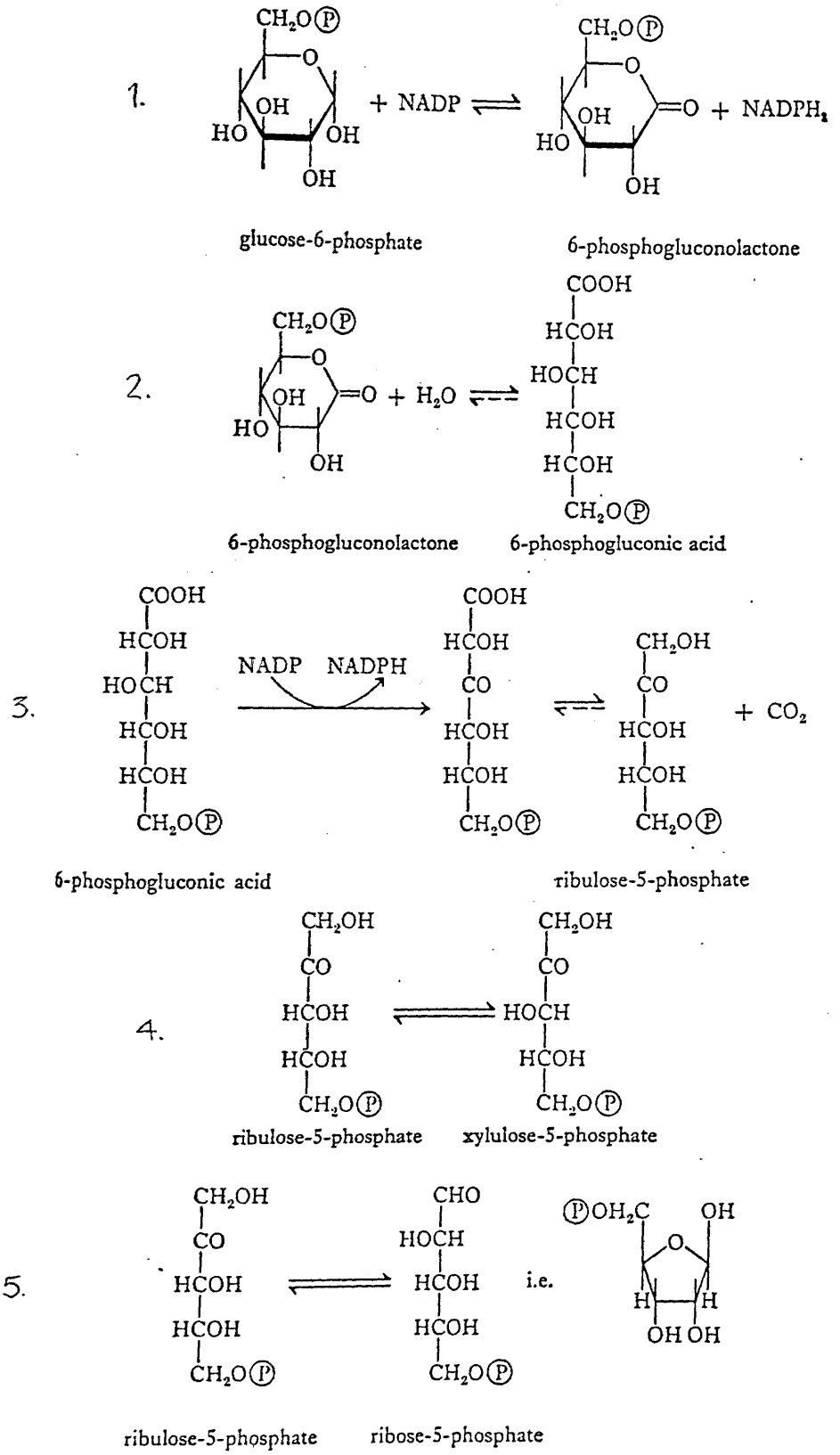


Figure 18. The hexose monophosphate (pentose phosphate) pathway.

this case an arbitrary value of 100% is assigned to a suspension of PMN prior to stimulation by opsonised latex particles.

The results of these tests provide additional information on the nature of the inhibition seen on treatment of normal human PMN with MMI and its putative metabolites.

5.1 Materials And Methods.

Determination Of The Effect Of Treatment With 10^{-3} M MMI, MTH, MH And MI On The Oxygen Consumption Of Resting And Latex-Stimulated PMN

Preparation of PMN and test compounds was as previously described (Chapter 3.1). The measurements of oxygen consumption were made using an oxygen electrode (Rank Brothers, Cambridge, England), the membrane of which was changed daily. Three ml of a PMN suspension containing 10^6 cells per ml, was pipetted into the chamber of the oxygen electrode, which was maintained at a constant temperature of 37°C. To this was added 300 µl of opsonised latex spheres, of 0.8 µm diameter, at a concentration of 10^9 per ml and the mixture was constantly stirred. Test runs contained 10^{-3} M MMI, MTH, MH, or MI. The amount of dissolved oxygen in the chamber was monitored over 60 minutes for oxygen depletion.

Determination Of The Effect Of 10^{-3} M MMI, MTH, MH And MI On The Hexose Monophosphate Shunt Activity Of Latex-stimulated Human PMN

Preparation of PMN and the test compounds was as previously

described (Section 3.1). The HMPS assay was performed in Warburg flasks in one ml volumes. Into each flask was put 100 µl of a PMN suspension containing 10^7 cells per ml PBS, 50 µl of fresh, autologous serum, 700 µl PBS (contains no glucose), 10 µl of either PBS or 0.1 M solution of the test compound and 50 µl of 1.4 X 10^{-8}M glucose-1-^{14}C (Amersham). One hundred µl of 5 N NaOH was pipetted into the centre well of the flask, containing a folded piece of Whatmans No.4 filter paper 0.5 X 6 cm. The side arm of 'stimulated' flasks received 100 µl of 0.8 µm diameter latex particles (Sigma) at a concentration of 10^9 per ml PBS. 'Unstimulated' flasks received 100 µl of PBS. The experiment was performed five to six times with each condition repeated in triplicate. To initiate the respiratory burst, the contents of the side arm were tipped into the main body of the flasks, which were tightly sealed with rubber stoppers, and incubated in an orbital incubator (Gallenkamp) at 37°C for two hours. The released $^{14}CO_2$ was "captured" by the NaOH in the centre well. At the end of the incubation period, the filter papers were removed and dried overnight. Ten ml scintillant was added to eachvial and the amount of radioactivity trapped was measured in a liquid scintillation spectrometer.

5.2 Results.

The oxygen consumption of unstimulated PMN was very low and was not found to be significantly affected by the addition of any of the compounds tested at a concentration of 10^{-3} M. Resting levels were between two and five percent of the

The Effect Of MMI, MTH, MH And MI On The Oxygen Consumption Of Latex-stimulated Human Polymorphonuclear Leukocytes.

Drug (10^{-3} M)	30 Minutes Incubation % Inhibition / Stimulation	X^2	P	60 Minutes Incubation % Inhibition / Stimulation	X^2	P
Methimazole	+49.01	8.53	0.05	+48.13	9.90	0.025
Methylthio-hydantoin	-13.81	1.29	N.S.	- 3.07	3.23	N.S.
Methylhydantoin	+ 1.94	4.95	N.S.	+ 0.77	1.93	N.S.
Methylimidazole	+ 5.50	2.16	N.S.	+58.34	17.16	0.05

Table 9

Table 9 shows the percentage inhibition/stimulation of oxygen consumption by latex-stimulated PMN. The percentage inhibition (-) or stimulation (+) is expressed as the difference between the drug-treated and control PMN over the control values. X^2-test statistics were applied to the data, assuming a Null Hypothesis that the drugs do not affect the oxygen consumption of latex-stimulated PMN, and that any differences observed are due to random sampling fluctuations. The X^2 values are shown, along with the P values. n=6.

arbitrary 100% value assigned to a suspension of PMN.

The oxygen consumption of stimulated PMN, however was markedly increased on addition of opsonised latex particles. The amount of oxygen consumed over a 60 minute period rose to between 30% and 40%.

The effect of the addition of 10^{-3} M MMI, MTH, MH and MI on the oxygen consumption of the stimulated cells is shown in Table 9.

Only MMI was shown to significantly stimulate the oxygen consumption of stimulated PMN after 30 minutes, and the increase was of the order of 50%. After 60 minutes, however both MMI and MI significantly stimulated the O_2 consumed by the PMN, again the increase was of the order of 50%. MH did not appear to affect the stimulated PMN at all and O_2 consumption levels were on a par with untreated stimulated PMN. MTH also had no significant effect, however in this case the observed increase in the amount of oxygen present in the PMN suspension during the first five minutes of stimulation did not reflect the pattern seen in the cases of MMI, MH and MI. On stimulation by latex particles, the amount of oxygenation of the suspension rose (respiratory burst), but after five to ten minutes as was the usual case, the oxygen did not appear to be depleted from the medium and instead the percentage of dissolved oxygen in the suspension dropped only slightly over 30 minutes, or not at all, thus

Effect Of MMI, MTH, MH And MI On The HMPS Activity Of Latex-Stimulated Human PMN

Drug	%age Stimulation	S.D.	X_2	P	n
Methimazole	19.87	9.59	23.02	0.001	5
Methylthiohydantoin	15.56	4.89	9.93	0.02 - 0.05	5
Methylhydantoin	13.81	1.00	6.41	0.1 - 0.2 (NS)	5
Methylimidazole	15.09	6.28	13.41	0.02 - 0.05	6

Table 10

Table 10 shows the mean $\pm$ standard deviation of the percentage stimulation of glucose-1-^{14}C oxidation by human PMN via the HMPS. The percentage stimulation is expressed as the difference between the drug treated and control PMN over the control values. X_2-test statistics were applied to the data, assuming a Null Hypothesis that the drugs do not significantly affect the oxidation of glucose via the HMPS, and that any differences observed are due to random sampling fluctuations. The X_2 values are shown, as is the P value indicating the probability of the null hypothesis being correct. All the drugs were used as a concentration of 10^{-3} M, and with the exception of methylhydantoin, all were found to cause significant stimulation of the HMPS.

after 60 minutes of incubation, the MTH-treated suspension contained between 95% and 105% dissolved oxygen.

The HMPS activity of resting PMN was not significantly affected by the presence of either MMI, MTH, MH or MI at 10^{-3} M. However the glucose oxidation of stimulated PMN was increased by MMI, MTH and MI at a concentration of 10^{-3} M, MMI-treatment resulting in around a 20% stimulation of the HMPS, and MTH and MI causing 15% stimulation over control levels (See Table 10). MH did not affect the glucose oxidation of stimulated PMN.

5.3 Discussion.

During the respiratory burst, an increase in oxygen consumption is noted in stimulated PMN. This oxygen is reduced by the PMN microbicidal oxidase system where NADPH is thought to be the immediate electron donor to the oxidase system. The activity of the HMPS, the main pathway of electrons to NADPH, is markedly increased in association with oxidase activity. Thus the two processes, oxygen consumption and glucose oxidation, are intimately bound. Impaired function of the HMPS has been shown to result in failure of oxidase activity (Gray et al, 1973).

Of the four compounds tested, only MMI exerted an effect on oxygen consumption following 30 minutes incubation with opsonised latex particles. MMI stimulated oxygen consumption

by 50%, suggesting that there would be a similar increase in the production of active oxygen species available to combat phagocytosed micro-organisms, thus boosting the PMN's microbicidal capacity. After 60 minutes incubation, both MMI and MI were responsible for a 50% increase in oxygen consumption. This could suggest that the imidazole part of the compounds is responsible for the increase in oxygen consumption. It is surprising that MTH did not significantly alter the rate or amount of oxygen consumption of the PMN, as it does inhibit the microbicidal function, but this may be due to the nature of MTH, which is an unstable compound in aqueous solution, oxidising rapidly at room temperature.

The HMPS activity of stimulated PMN was shown to be significantly stimulated by MMI, MTH and MI by between 15% and 20%, MH did not significantly affect glucose oxidation. It would be expected that if oxygen consumption by the PMN was increased, the HMPS activity also would be stimulated.

Tsan and McIntyre (1975) showed that PTU and MMI, at similar concentrations used in this study, caused stimulation of glucose-1-^{14}C oxidation by stimulated human PMN by between 50% and 100% for PTU at a concentration of 10^{-4} M and 61% for MMI also at 10^{-4} M. They similarly found HMPS activity in unstimulated cells to be unaffected by PTU and MMI, however on addition of H_2O_2 to the resting cells, an increase in HMPS activity was noted and they concluded that H_2O_2 and not phagocytosis was

responsible for the stimulation of glucose oxidation. This could provide an explanation for the results observed here. MMI is known to inhibit thyroid peroxidase, and the slight but consistent inhibition of bacterial killing shown here is thought to be as a result of impaired myeloperoxidase activity. Therefore if the MPO was inhibited, H_2O_2 would not be used up by the cells as normal, in the production of HOCl, it would instead by used by the glutathione peroxidase system and remain to stimulate the HMPS. Thus more glucose oxidation would occur, being detected as $^{14}CO_2$. The lower values of 15%-20% stimulation obtained with 10^{-3} M MMI in comparison to the figure of 61% with 10^{-4} M found by Tsan and McIntyre (1975) may be as a result of the different methods of detecting labelled CO_2.

Tsan and McIntyre (1975) examined the effect of a number of compounds on HMPS activity of stimulated PMN, such as PTU, MMI, thiouracil, thiourea, uracil, urea and adenine. They conclude that a minimal common structure of "thiourea", or a thiourylene group was necessary for this stimulatory effect. The results shown here indicate that the thiol group is not necessary to cause stimulation of HMPS activity, as MI is responsible for a similar amount of O_2 consumption and glucose oxidation as MMI. Possession of either the imidazole or the thiol group may be enough, as MTH also stimulated glucose oxidation. MH had no effect on either oxygen consumption or glucose oxidation and is known not to affect microbial killing by the PMN. Therefore, although these

parameters of function are thought of as reliable indicators of the level of microbicidal function of the PMN, careful interpretation is necessary, as although O_2 consumption is stimulated by MMI, a similar increase in microbicidal function was not seen.

CHAPTER 6

The Effect Of MMI And Its Putative Metabolites On The Release And Activity Of PMN Lysosomal Enzymes: Myeloperoxidase, Lysozyme, Alkaline Phosphatase And Lactoferrin.

6.0 Introduction.

The 'killing apparatus' of the PMN consists of oxidative and non-oxidative-components. The oxidative mechanisms and the effect of MMI, MTH, MH and MI have been examined, in order to examine the actions of the compounds MMI, MTH, MH and MI on the non-oxidative aspect of the PMN's killing capacity, human PMN were stimulated to release lysosomal enzymes. These enzymes include myeloperoxidase, lysozyme, alkaline phosphatase and lactoferrin. The effect of MMI, MTH, MH and MI on the activity of these enzymes has been assessed.

6.1 Materials And Methods.

Stimulation Of PMN To Release Lysosomal Enzymes

The PMN were isolated as before and suspended in HBSS (Gibco) to a final concentration of 2 X 10^6 per ml. Cytochalasin B (Sigma) at a concentration of 5 μg/ml and 10^{-6} M N-f-mlp were added to one ml of the PMN suspension and incubated at 37^{o}C with shaking for 15 minutes. The PMN were sedimented by centrifugation at 1500 g for two minutes at 4^{o}C. The supernatant containing the released lysosomal enzymes was withdrawn and stored at -20^{o}C, where it was stable for at least one month.

Effect Of MMI, MTH, MH And MI On The Release Of Lysosomal Enzymes

Concentrations of MMI, MTH, MH and MI ranging from 10^{-7} to 10^{-3} M were pre-incubated with PMN for 30 minutes before being included in the reaction mixture consisting of PMN, cytochalasin B and f-mlp as above. The released enzyme preparation was assayed immediately for its MPO content spectrophotometrically as described below. The procedure was performed in duplicate with three separate samples of PMN from different donors.

Measurement Of Myeloperoxidase Activity

The reaction mixture consisted of PMN supernatant containing released lysosomal enzymes, 1.6 mM tetramethylbenzidine (TMB) (Sigma), 0.3 mM H_2O_2, 80 mM sodium phosphate buffer (pH 5.4) 8% N, N-dimethylformamide and 40% PBS in a total volume of 500 µl. A range of volumes of PMN supernatant was assayed to establish optimal assay conditions. The volumes of PMN supernatant ranged from 10 µl to 100 µl. The mixture was incubated at 37°C with shaking for times between one and fifteen minutes. At the end of the incubation period, the tubes were immersed in an ice-bath and 1.75 ml of 200 mM sodium acetate buffer (pH 3.0) was added to terminate the reaction. The MPO product was measured in a Unicam SP 500 spectrophotometer at a wavelength of 655 nm. The effect of the various compounds at concentrations of between 10^{-7} and 10^{-3} M was assessed by including them in the reaction mixture.

Assay Of Lysozyme In Human PMN, And The Effect Of MMI, MTH, MH And MI.

The preparation of PMN and drugs was as before. Micrococcus lysodeikticus (Sigma) was grown overnight in 2.5% nutrient broth (Difco) at 37°C. One loopful of the broth culture was streaked down the centre of a nutrient agar plate and swabbed over the entire surface of the plate to produce a uniform distribution of organisms. PMN suspended in PBS to give a concentration of 10^7/per ml were pre-incubated with concentrations of drug ranging from 10^{-7} to 10^{-3} M for 10 minutes at 37°C. Following this pre-incubation, 20 µl of the PMN/drug mixture was dropped onto the nutrient agar plates lawned with M.lysodeikticus, four drops per plate, this was performed in triplicate. The plates were incubated overnight at 37°C. The zones of inhibition of growth of M.lysodeikticus caused by the lysozyme content of the PMN were measured. The zone diameters of drug treated and control PMN were compared.

Assay Of Egg White Lysozyme Activity And The Effect Of MMI, MTH, MH And MI.

The assay procedure was as described on the Sigma bulletin accompanying lysozyme Product No.L-6876: into a quartz cuvette was pipetted 2.5 ml of a Micrococcus leisodeikticus suspension in 0.066 M potassium phosphate buffer (pH 6.2). The A_{450} of the suspension was between 0.6 and 0.7. To this was added 0.1 ml of a lysozyme solution (Sigma) containing

200 to 400 U per ml. The cuvette was immediately inverted to mix the contents and the decrease in absorbancy was charted.

Alkaline Phosphatase Assay

Five hundred µl each of 2-amino-2-methyl-1-propanol buffer (Sigma) and p-nitrophenylphosphate (Sigma) were incubated at 37°C in a water bath to equilibrate. To the blank tube was added 100 µl of distilled water or concentrations of drug from 10^{-7} - 10^{-3} M, to the control tubes was added 100 µl of PMN supernatant (containing released lysosomal enzymes), and to the test tubes was added 100 µl of PMN plus either MMI, MTH, MH or MI at concentrations between 10^{-7} and 10^{-3} M. The tubes were mixed gently and placed in a water bath at 37°C for 15 minutes. At the end of the incubation period 10 ml of 0.05 N sodium hydroxide was added to each tube. The optical density was read at 400-420 nm in silica glass cuvettes.

Lactoferrin Assay

Preparation Of Micro-organisms

A light innoculum of S. aureus and S. pyogenes was grown up overnight in 2.5% nutrient broth (Difco). The cultures were centrifuged and the pellet resuspended in 1.0 ml of phosphate buffered saline. Five hundred µl of this was used as the innoculum for a 100 ml culture, which was incubated at 37°C

under conditions of constant shaking in a Gallenkamp orbital incubator for 2-3 hours. Growth curves had previously been constructed for both organisms, and the incubation time was chosen to ensure that the bacterial cells were in the logarithmic phase of growth. The cultures were centrifuged at 1500 X *g* and the pellet washed three times in PBS and resuspended to give a concentration of 2.0×10^9 colony-forming units (c.f.u) per ml.

Preparation Of Apo-lactoferrin And Iron-saturated Lactoferrin

Purified human lactoferrin (Sigma) was suspended in PBS at a concentration of 1.0 mg per ml. The apo- (iron-free) lactoferrin was prepared by dialysis against 0.1 M citric acid (pH 2.3), the iron-saturated lactoferrin by dialysis against saturated ferrous ammonium sulphate. Each was then dialysed against double distilled water.

Assay Of Lactoferrin Activity

In sterile bijoux, 100 μl of the bacterial suspensions were incubated with 200 μl of various concentrations of lactoferrin, (0 to 1 000 μg per ml) for one hour at 37°C. After this incubation time portions of the incubation mixture were serially diluted and plated onto chocolate agar plates (lysed horse blood) and incubated for 18 hours at 37°C. The number of colonies on each plate were counted, and the mean c.f.u per ml worked out.

6.2 Results.

The Effect Of MMI, MTH, MH And MI On The Release Of Lysosomal Enzymes (As Assessed By The Release Of MPO)

It was found that the compounds tested did not significantly affect the release of MPO from stimulated PMN by the method used.

The Effect Of MMI, MTH, MH And MI On MPO Activity

The optimum amount of lysosomal enzyme preparation used in the assay was found to be 100 µl, which enabled the showing of dose-related inhibition by the compounds over a wide range of concentrations.

The optimum incubation time for the MPO assay was found to be 12 minutes, after this time no further development of colour was observed.

It was found that MMI, MTH and MI caused dose-related inhibition of MPO activity whereas MH did not significantly affect MPO activity. The compounds affected MPO activity to different degrees, with MTH causing the greatest amount of inhibition, followed by MMI and MI. The compounds at concentrations of 10^{-6} and 10^{-7} M did not affect MPO activity as measured by this assay. The inhibition seen was not overcome by addition of excess H_2O_2. (Table 11)

Table 11

Table 11 shows the percentage inhibition of MPO activity, as measured spectrophotometrically, caused by various concentrations of MMI, MTH, MH and MI. Correlation coefficient statistics were applied to the results and the r values are shown, along with this converted to a t value (Student's t-test) and the P value.

The assay was performed in triplicate and the results shown represent the mean of three separate determinations.

NS - not significant

Table 11

Effect Of MMI, MTH, MH And MI On MPO Activity

Drug And Conc.	% Inhibition Of MPO Activity	r Value	t Value	P Value	n
Methimazole					
10^{-3} M	48.92	-0.669	3.486	P=0.01	18
10^{-4} M	41.40				
10^{-5} M	10.22				
Methylthiohydantoin					
10^{-3} M	100.00	-0.797	5.278	0.001	18
10^{-4} M	59.14				
10^{-5} M	2.00				
Methylhydantoin					
10^{-3} M	0.00	-0.176	0.669	N.S.	18
10^{-4} M	3.23				
10^{-5} M	0.00				
Methylimidazole					
10^{-3} M	36.56	-0.493	2.267	0.05	18
10^{-4} M	20.43				
10^{-5} M	0.00				

FIGURE 19

LEGEND

● - ● MMI

■ - ■ MTH

○ - ○ MH

▲ - ▲ MI

Each point represents the mean ± standard deviation of three separate determinations.

Figure 19

Effect of concentrations of MMI, MTH, MH and MI between 10^{-3} and 10^{-5} M on MPO activity. Compound concentration versus optical density measured at 655nm.

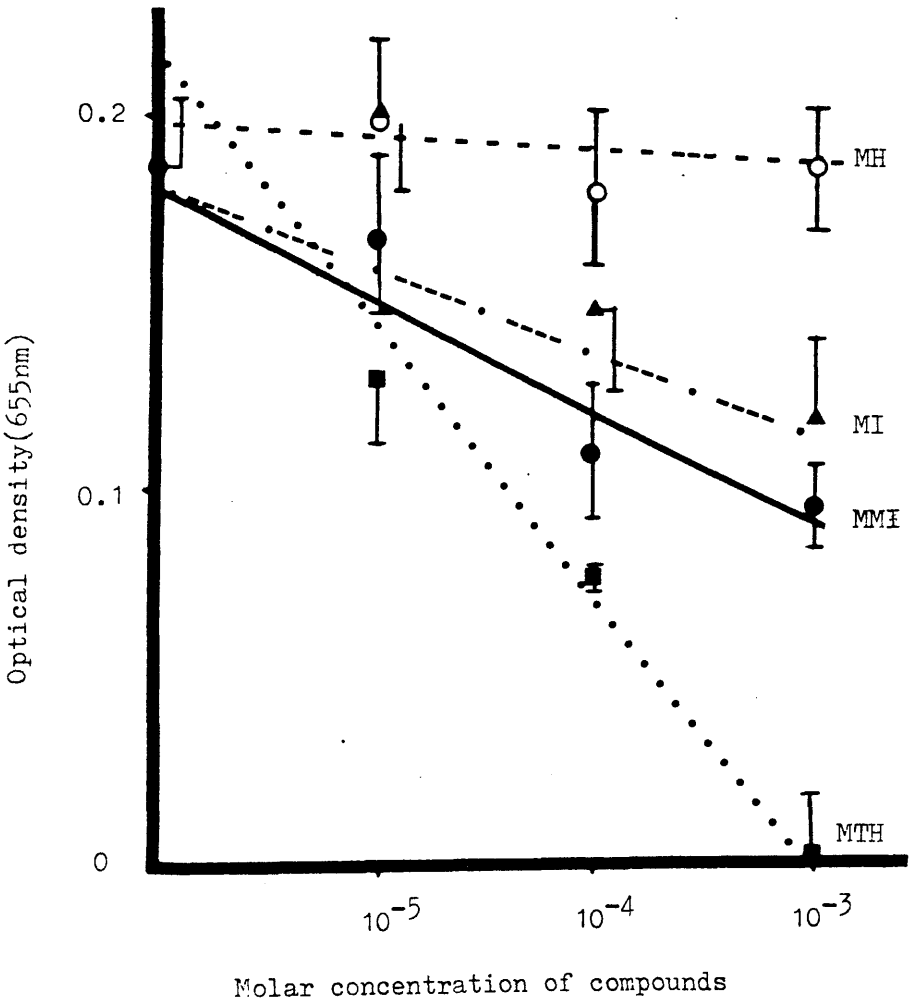


Table 12

Table 12 shows the effect of the various compounds on the lysozyme activity of human PMN. The zone diameters recorded represent the mean of three separate determinations and the standard deviations are also shown.

Table 12

Effect Of MMI, MTH, MH And MI On The Lysozyme Activity Of Human PMN

Log Conc. Compound	Zone Diameter (mm)	Standard Deviation
Methimazole		
0	7.00	0.00
-5	7.33	0.58
-4	7.00	0.00
-3	7.17	0.29
Methylthiohydantoin		
0	6.67	0.58
-5	7.00	0.00
-4	7.00	0.00
-3	6.67	1.15
Methylhydantoin		
0	6.75	0.96
-5	6.75	0.50
-4	6.75	0.29
-3	6.88	0.25
Methylimidazole		
0	7.33	0.58
-5	7.33	0.58
-4	7.00	0.00
-3	7.33	1.15

The percentage inhibition caused by the various compounds are shown in table 11.

Effect Of MMI, MTH, MH And MI On Human PMN Lysozyme Activity (Assayed In Whole Human PMN)

The compounds at concentrations in the range 10^{-5} to 10^{-3} M did not significantly affect the lysozyme activity of human PMN as assayed by the clearance of M. leisodeiktikus from agar plates. The zone diameters of the areas cleared by the human PMN were not significantly different in control and drug treated PMN. It was noted that the cleared zone diameter was proportionally related to the amount of PMN applied to the agar plates lawned with M. leisodeiktikus. (Table 12)

Effect Of MMI, MTH, MH And MI On The Activity Of Hen Egg-white Lysozyme And The PMN Lysozyme From The Released Lysozomal Enzymes

It was found that the compounds tested at concentrations between 10^{-5} and 10^{-3} M did not affect the activity of either the egg-white or PMN lysozyme as measured by a drop in optical density of a suspension of M. leisodeiktikus.

Effect Of MMI, MTH, MH And MI On Alkaline Phosphatase Activity

MMI and MTH inhibited alkaline phosphatase activity in a dose-related fashion. Correlation coefficient statistics were applied to the data and r values of -0.393 and -0.496 were

FIGURE 20.

LEGEND

● - ● MMI

■ - ■ MTH

Each point represents the mean ± standard deviation of three separate determinations.

Figure 20

Effect of MMI and MTH on PMN alkaline phosphatase activity.

Molar concentration of compound versus optical density function measured at 420nm.

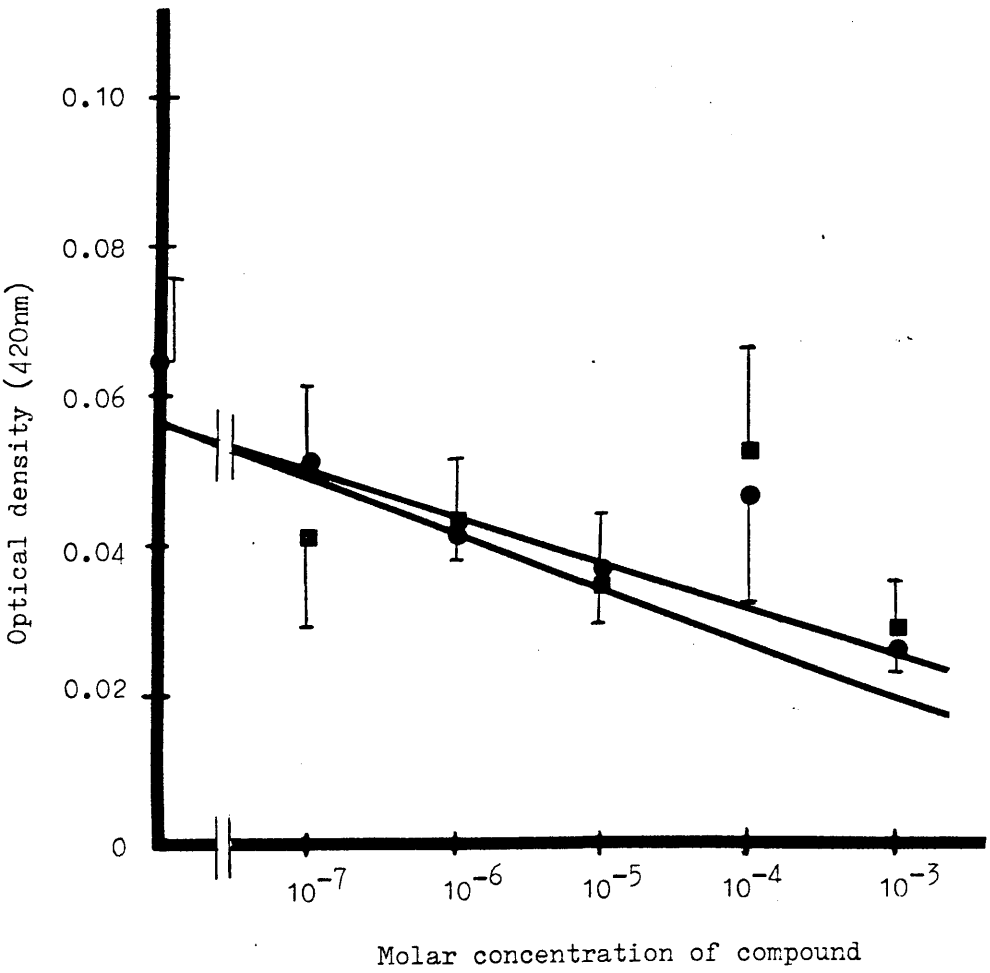


FIGURE 21

LEGEND

● - ● S.aureus

■ - ■ S.pyogenes

Growth curves of S.aureus and S.pyogenes

Optical density versus time in hours

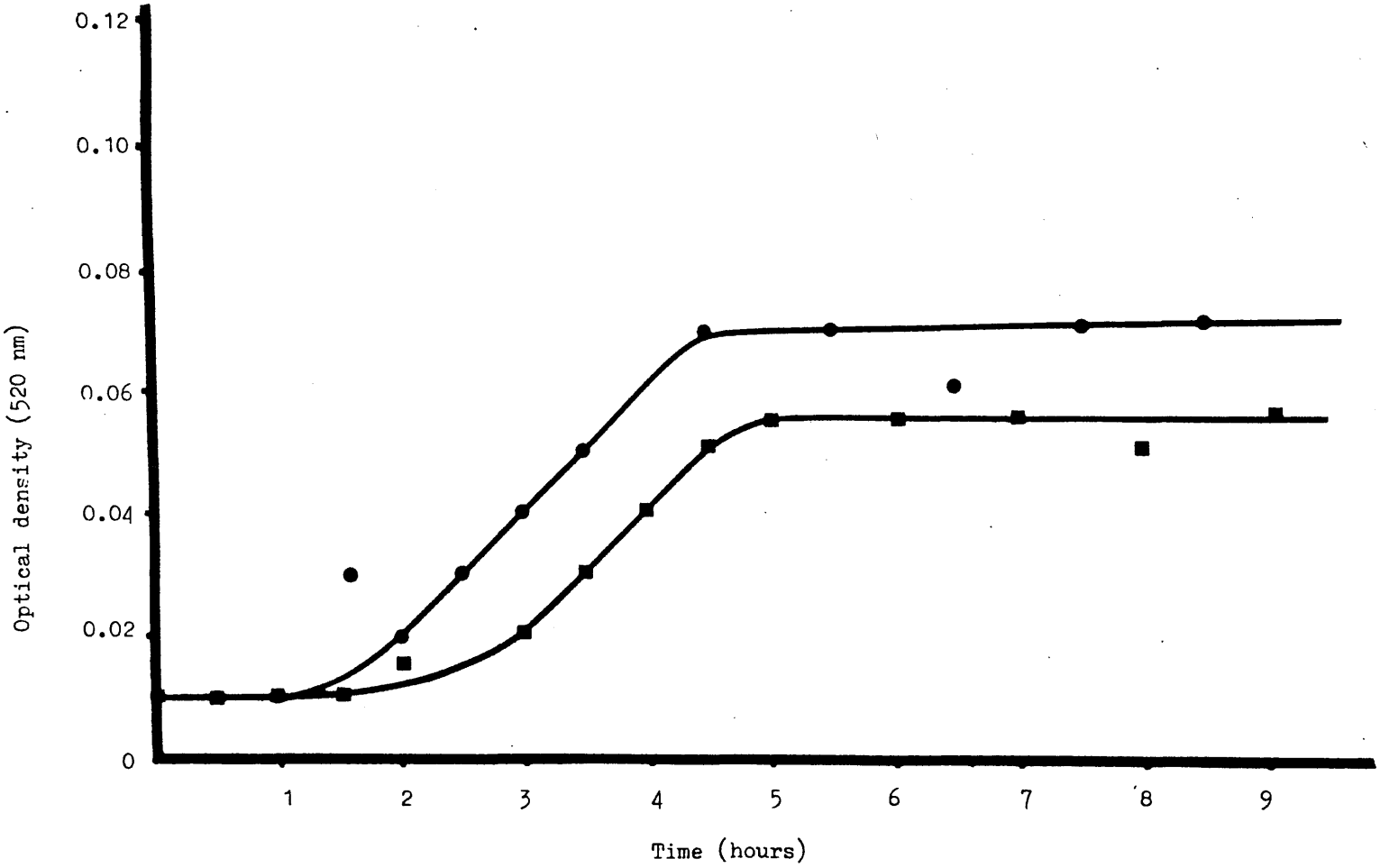


Figure 21

FIGURE 22

LEGEND

● - ● S.aureus

■ - ■ S.pyogenes

Influence of apo-lactoferrin on the survival of S.aureus and S.pyogenes

Number of surviving micro-organisms versus lactoferrin concentration

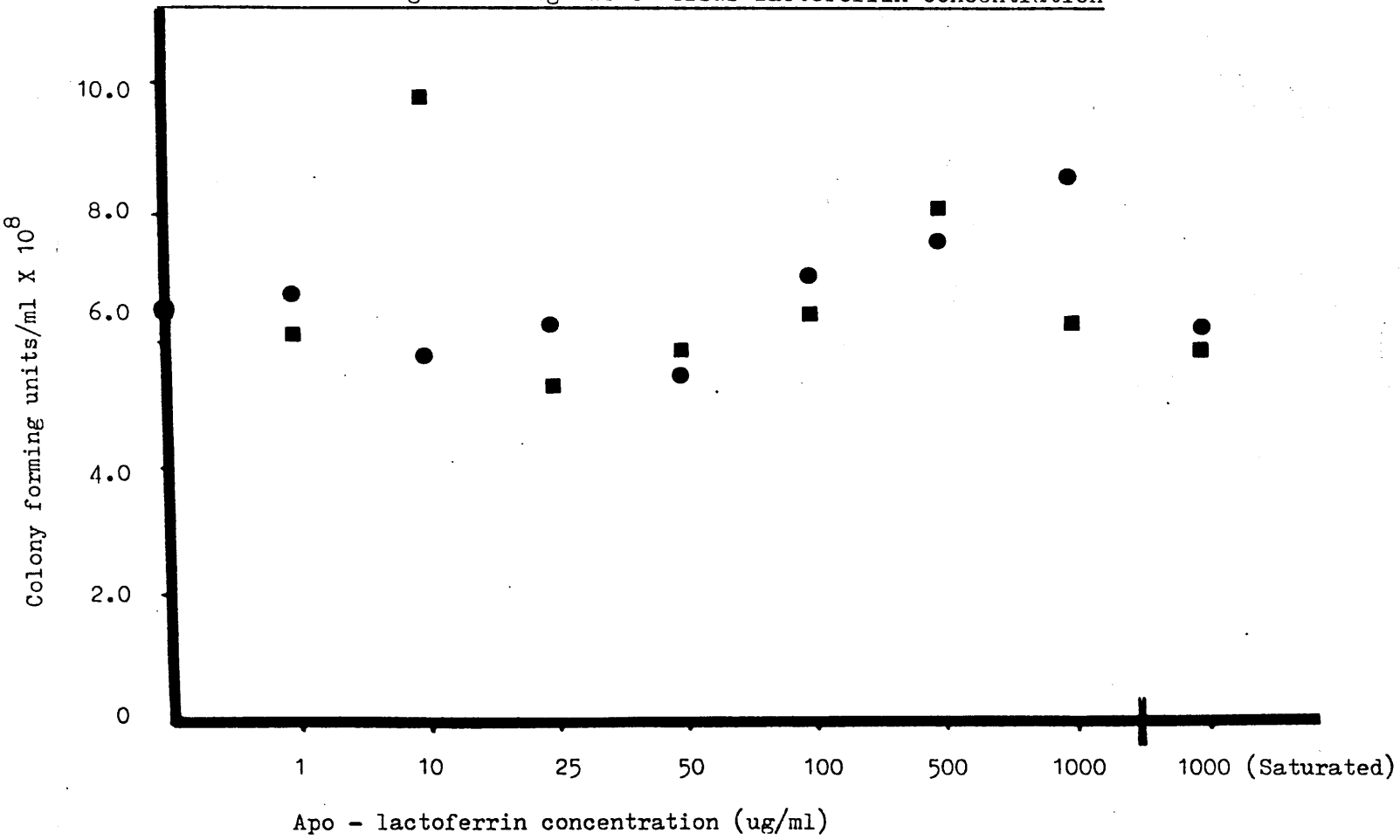


Figure 22

found for MMI and MTH; these correspond to P value of 0.05 and 0.01 respectively, indicating a high probability of MMI and MTH being responsible for the inhibition shown. MH did not significantly affect alkaline phosphatase activity, and MI was shown to significantly affect activity at a concentration of 10^{-3} M only. Student's t-test statistics were applied to the results and a t-value of 2.174 was found, the corresponding P value is 0.05.

Results Of Lactoferrin Assay

Concentrations of lactoferrin ranging from 0 to 1000 ug per ml were incubated with S.aureus and S.pyogenes, however, none of the concentrations appeared to have a cidal effect on the organisms treated, and there was no apparent dose-related response, therefore it was concluded that lactoferrin may have a role to play in the bactericidal action of the PMN, and the effect, if any of MMI, MTH, MH and MI was not assessed.

Measurement Of The pH Of The Compounds Used In Enzyme Assays

The compounds were made up in PBS which had a pH of 7.4. Solutions of methimazole of concentrations from 10^{-1} to 10^{-7} M had a pH of 7.45. 10^{-1} M methylthiohydantoin had a pH of 7.35, however solutions of 10^{-2} to 10^{-7} had a pH of 7.4. Methylhydantoin at all concentrations had a pH of 7.4. Methylimidazole's pH at 10^{-1} M was 9.0 but this dropped to 7.7 at 10^{-3} M and to 7.4 from 10^{-4} to 10^{-7}.

6.3 Discussion.

The mechanisms of the microbicidal function of PMN have been extensively investigated by many groups over the last twenty years. The generation of reduced oxygen products and the release of lysosomal enzymes are responsible for them. However in recent years a great deal of attention has been focused on the oxidative side of the PMN's killing capacity. The non-oxidative role is one which should not be overlooked and the interaction between oxidative and non-oxidative mechanisms remains to be thoroughly investigated.

On stimulation by particulate or non-particulate agents, such as PMA and f-mlp, PMN are induced to release their lysosomal enzymes. The effect of MMI and its putative metabolites was investigated, as SH- containing compounds are known to be capable of modulating PMN response (Rajkovic and Williams, 1984). However, they were found not to alter enzyme release, therefore this can be ruled out as being a factor in the inhibition of the PMN's microbicidal activity. MMI-treated PMN therefore appear to degranulate normally. The effect of MMI, MTH, MH and MI on these enzymes' functions could then be assessed.

Myeloperoxidase is a dimeric enzyme, containing two haem prosthetic groups (Agner, 1958) which bind small anions (Eglinton *et al*, 1982; Wever and Bakkenist, 1980) and react

with H_2O_2 (Harrison et al, 1980). It is fundamentally unique in its capacity to catalyse the peroxidation of chloride ion (Harrison and Schultz, 1976). The reaction produces hypochlorous acid, whose ability to cause extremely rapid oxidative degradation of a wide variety of biological substrates, including porphyrins, haems and haem proteins (Albrich et al, 1981), suggests that it is the ultimate MPO-generated toxin (Klebanoff and Clark, 1978). MMI and MTH were found to inhibit human MPO activity in a dose-related manner at concentrations between 10^{-5} and 10^{-3} M, MI also inhibits MPO activity, but to a lesser extent, and MH has no significant effect. The pattern of inhibition reflects that of bacterial killing, providing more evidence of the crucial role played by MPO in the destruction of micro-organisms.

Klebanoff and Hamon (1972) found that MMI inhibited peroxidase-catalysed iodination by intact leukocytes, and they suggested that iodination reaction is to some extent a measure of MPO and H_2O_2 in the intact cell. The results shown here also suggest that MMI and its metabolites MTH and MI inhibit myeloperoxidase-catalysed reactions. There are a number of possible ways in which this inhibition could occur. The MMI could act as a substrate for MPO, and bind or link closely with the enzyme, competing with H_2O_2 for binding sites, or with MPO-H_2O_2 for reaction with Cl^-. Alternatively it could react reversibly or irreversibly

with MPO, inactivating it somehow, eg. by modifying part of the enzyme's active site or prevent the MPO-H_2O_2 from reacting with Cl^- to form HOCl. A further possibility is that MMI acts on or is acted upon by HOCl.

Addition of excess H_2O_2 did not overcome the inhibition seen, which suggests that the compounds do not act as competitive inhibitors. From the results of the HMPS assay, it was concluded that H_2O_2 was probably not being used up normally by the cell, instead taking part in alternative oxidation reactions, and via the glutathione peroxidase system, stimulating HMPS activity. In the presence of MMI, MTH and MI at 10^{-3} M concentrations, HMPS activity was significantly stimulated. The amount of H_2O_2 and other active oxygen species produced by stimulated PMN is probably increased in the presence of MMI and MI, as oxygen consumption was doubled. Therefore it seems likely that MMI, MTH and MI inhibit MPO to differing degrees by binding to the enzyme in such a way as to prevent further reaction with H_2O_2 or Cl^-. Thyroid peroxidase is thought to be inhibited by MMI in a similar way, by a reaction between MMI and the oxidised haem group produced by interaction between TPO and H_2O_2 (Engler et al, 1982).

Klebanoff and Pincus (1971) suggested that inhibition or absence of MPO resulted in increased utilisation of H_2O_2

in non-MPO-mediated H_2O_2-dependent reactions such as formate oxidation and HMPS activation. They also considered the possibility that microbicidal activity of MPO is offset in part by an increase in the non-enzymatic microbicidal activity of H_2O_2.

Lysozyme was found to be unaffected by any of the compounds tested. It is one of a class of enzymes that hydrolyse bacterial cell walls (Salton, 1952) and as such is probably more important in the degradation of phagocytosed micro-organisms than in killing.

Alkaline phosphatase, which catalyses the hydrolysis of organic phosphate esters was inhibited by MMI, MTH and MI. The inhibition was of the order of 60% at 10^{-3} M concentrations, and was dose-related. Neutrophil alkaline phosphatase activity may serve as a very sensitive index of a discrete inflammatory state not reflected in the physical state of a patient. The inhibition of alkaline phosphatase activity by MMI and its metabolites reflects the decrease in microbicidal activity of the PMN and as such MMI may be thought of as an anti-inflammatory compound. Imidazole compounds have been implicated in cases of liver damage. One report claims MMI to be responsible for inducing drug-hepatitis in an unstated number of their hyperthyroid cases, 30% of the patients had altered serum alkaline phosphatase activity (Hackenberg

et al, 1971). The mechanism of action remains to be examined.

Lactoferrin does not have any effect on the viability of the S.aureus or S.pyogenes strains tested here, and it was concluded that lactoferrin alone probably plays a very minor, if any role in the microbicidal function of the human PMN, Leffell and Spitznagel (1975) reported that 80% of the lactoferrin discharged during phagocytosis is secreted into the extracellular media rather than into the phagosome, suggesting that lactoferrin may exert its effects primarily in the extracellular location. Wang-Iverson et al (1978) reported that cells depleted of specific granules by pre-treatment with PMA retained significant bactericidal capacity when the particle to cell ratios were low, implying that lactoferrin as well as other products of specific granules may be of secondary importance in the microbicidal reactions of PMN.

CHAPTER 7

THE EFFECT OF FOSFESTROL ON SELECTED PARAMETERS OF PMN FUNCTION

7.0 Introduction.

Fosfestrol (Fig 23) is a synthetic, non-steroidal, water-soluble oestrogen compound, used clinically in the treatment of prostatic carcinoma. Oestrogenic compounds are known to exert an effect upon various parameters of the immune response. Studies with PMN indicate that both steroidal and non-steroidal compounds stimulate the bactericidal and fungicidal activity of human and animal PMN (Mitchell and Sbarra, 1965, 1966; Jacobs et al, 1973; Sulwicz et al, 1981).

Peroxidase has an oxidation potential of approximately 1,000 mV, for the compound I to compound II couple. Therefore, this enzyme should have the capacity of oxidising MMI, PTU and fosfestrol, all of which have oxidation potentials in the region 450-600 mV. This suggests that the action of thiourylene drugs and fosfestrol on PMN may be related at least in part to their oxidation potential. Oestrogens are also known to serve as a substrate for peroxidase (Ball and Knuppen, 1980). There are no oestrogen high affinity receptors in leukocytes (Personal communication from Dr M.M. Ferguson).

Therefore the effect of fosfestrol on selected parameters of

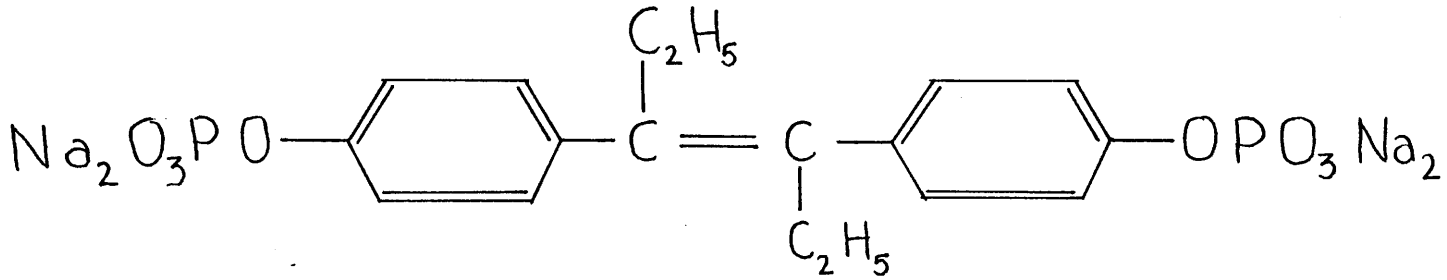


Fig. 23 Structure of Fosfestrol.

PMN function was investigated, using a range of concentrations.

7.1 Materials And Methods.

The techniques used to investigate chemotaxis, phagocytosis, microbicidal capability, oxygen consumption, HMPS activity, lysozyme, alkaline phosphatase and myeloperoxidase activity were as described in previous chapters.

Electron Microscopy

Two hundred and fifty µl of a PMN suspension containing 8 X 10^7 cells/ml was placed in a BEEM 00 capsule with a conical tip. To this was added 250 µl of a Candida albicans suspension containing 8 X 10^8 cells/ml and either 5 µl of 0.1 M fosfestrol (W.B. Pharm. Ltd.) in HBSS or HBSS (pH 7.3). The PMN/Candida mixture was incubated at 37°C with shaking for 30 or 60 minutes. At the end of the incubation period the tubes were centrifuged at 110 g to sediment the PMN, the supernatant was removed and the PMN pellet was fixed in phosphate-buffered 3% glutaraldehyde, pH 7.5, for 3 hours at room temperature. After fixation the glutaraldehyde was carefully pipetted off, leaving the sediment as a moderately firm pellet in the tip of the capsule. Processing was carried out in the capsule rather than cause possible disruption and damage to the cells by transferring them to another vessel. The PMN pellet was washed in several changes of Millonig's phosphate buffer over a period of 12 hours and then post-fixed in 1% osmic

acid in Millonig's phosphate buffer for 1½ hours at room temperature. Further rinsing in buffer over a three hour period was followed by dehydration through ascending grades of ethanol, which was completed by four changes of absolute ethanol of ½ hour duration each. The pellet was cleared in two changes of propylene oxide each of ½ hour duration, it was next soaked in propylene oxide/Araldite resin mixture of equal amounts for 6 hours, then put into propylene oxide/ Araldite resin 25/75 for 12 hours, before being soaked in pure Araldite resin mixture for a further 12 hours. Finally fresh Araldite resin mixture was added to the capsule which was placed in an oven at 45°C for 12 hours; the temperature was raised to 60°C for 15 hours to complete polymerisation of the resin.

Care was taken to ensure that both test and control specimens received identical treatment. Survey semi-thin sections 0.5-1.5 um thick were cut on a Reichert Autocut and stained with Azur Blue II. Areas were selected and thin sections cut on an L.K.B. ultotome. Grids with thin sections were stained for 5 minutes with a 50% ethanolic solution of uranyl acetate, then after washing in distilled water and drying, stained in a lead citrate solution based on the Reynolds (1963) method. Sections were examined in a Jeol 100S transmission electron microscope.

7.2 Results

Tables 13 to 16 show the effect of fosfestrol on the chemotaxis, random movement, phagocytosis and intracellular killing of micro-organisms by PMN.

7.2 Results.

Effect Of Fosfestrol On The Chemotaxis And Random Movement Of Human PMN With MEM As Chemo-attractant

Table 13

Log conc^{n} fosfestrol	$\bar{x}A \pm s$	$\bar{x}B \pm s$	$\bar{x}A/B \pm s$	$\bar{x}(A-B) \pm s$	r value A/B	A-B
0	1.58 ± 0.14	1.50 ± 0.00	1.06 ± 0.10	0.08 ± 0.14		
-3	1.58 ± 0.14	1.58 ± 0.14	1.00 ± 0.00	0.00 ± 0.00	N.S.	NS
-4	1.50 ± 0.00	1.50 ± 0.00	1.00 ± 0.00	0.00 ± 0.00		
-5	1.67 ± 0.29	1.67 ± 0.29	1.00 ± 0.00	0.00 ± 0.00	0.122	0.193

Table 13

Table of the linear distance travelled by PMN away from (B) and towards (A) the control substance MEM (minimal essential medium). Correlation coefficient statistics were applied to the chemotactic index (A/B) and differential (A-B) figures. The r values were not significant. The figures represent the mean $\pm$ standard deviation of 3 separate determinations performed in triplicate.

Effect Of Fosfestrol On The Chemotaxis And Random Movement Of Human PMN With Zymosan-activated Serum As Chemo-attractant

Table 14

Log conc^{n} fosfestrol	$\bar{x}A \pm s$	$\bar{x}B \pm s$	$\bar{x}A/B \pm s$	$\bar{x}(A \pm B)-s$	r value A/B	A-B
0	3.50 ± 1.32	1.50 ± 0.00	2.33 ± 0.88	2.00 ± 1.32	-0.200	-0.200
-3	3.50 ± 0.87	1.33 ± 0.29	2.67 ± 0.58	2.17 ± 0.76	NS	NS
-4	3.17 ± 0.76	1.17 ± 0.29	2.89 ± 1.17	2.00 ± 1.00		
-5	2.67 ± 0.29	1.17 ± 0.29	2.33 ± 0.29	1.50 ± 0.00		

Table 14

Table14 shows the same data as Table13 except that instead of MEM, the PMN travelled away from and towards zymosan-activated serum.

Table 15

Table of mean ± standard deviation of the reduction in numbers of organisms over time expressed as the percentage reduction of original inoculum over 30, 60 and 120 minutes. The figures represent the mean of 3 separate determinations performed in triplicate. Correlation coefficient statistics were applied to the results, however no significant dose-related effect was seen due to fosfestrol.

Table 15

Effect Of Fosfestrol On The Phagocytosis Of Various Organisms

Percentage Reduction In Numbers Of Organisms

Log drug conc^{n}/ organism	30 minutes incubation	r value	60 minutes incubation	r value	120 minutes incubation	r value
S.aureus	$\bar{x}\pm s$		$\bar{x}\pm s$		$\bar{x}\pm s$	
0	97.45 ± 0.69		98.36 ± 0.10		98.73 ± 0.20	
-3	95.64 ± 1.21	NS	98.00 ± 0.05	NS	98.18 ± 0.13	NS
-4	97.82 ± 0.53		98.18 ± 0.19		98.36 ± 0.21	
S.pyogenes						
0	97.52 ± 0.24		99.00 ± 0.20		98.64 ± 0.07	
-3	98.57 ± 0.31	NS	98.57 ± 0.14	NS	97.38 ± 0.12	NS
-4	98.67 ± 0.19		98.61 ± 0.13		98.29 ± 0.04	
E.coli						
0	98.63 ± 0.15		97.93 ± 0.09		92.22 ± 0.25	
-3	97.63 ± 0.20	NS	97.70 ± 0.11	NS	94.81 ± 0.61	NS
-4	98.52 ± 0.13		97.52 ± 0.04		93.70 ± 0.39	
L.carei						
0	96.64 ± 0.07		98.45 ± 0.07		99.07 ± 0.04	
-3	94.26 ± 1.02	NS	98.42 ± 0.25	NS	97.83 ± 1.13	NS
-4	95.33 ± 2.73		97.66 ± 0.76		98.01 ± 0.89	
C.albicans						
0	98.00 ± 0.24		99.54 ± 0.13		99.80 ± 0.08	
-3	99.76 ± 0.13	NS	99.79 ± 0.17	NS	99.80 ± 0.09	NS
-4	99.10 ± 0.18		99.71 ± 0.15		99.80 ± 0.09	

Table 16

Table of the mean $\pm$ standard deviation of the reduction in numbers of organisms expressed as the percentage reduction of original innoculum over 30, 60 and 120 minutes. The figures represent the mean of 4-6 separate determinations performed in triplicate. Correlation coefficient statiscis were applied to the results, the r value and P value are shown.

*Not done

Table 16

Effect Of Fosfestrol On The Intracellular Killing Of Various Organisms By Human PMN

Percentage Reduction In Numbers Of Organisms

Log drug conc^{n}/ organisms	30 minutes incubation	r value	60 minutes incubation	r value	120 minutes incubation	r value
S.aureus	$\bar{x}\pm s$		$\bar{x}\pm s$		$\bar{x}\pm s$	
0	73.99±4.77	-0.732	79.52±4.64	0.131	90.24±5.11	0.028
-3	59.28±15.16		79.71±10.42		89.93±6.64	
-4	77.79±4.26	P 0.05	77.93±10.39	NS	90.39±0.54	NS
-5	73.73±0.18		78.55±0.13			
S.pyogenes						
0	57.98±14.12		71.73±6.47		79.73±8.24	
-3	47.36±15.92	-0.129	67.18±10.89	0.444	86.33±3.13	0.224
-4	53.40±11.32	NS	89.91±1.39	NS	85.73±3.05	NS
-5	57.10±13.90		70.20±6.20			
E.coli						
0	44.17±5.00		90.54±1.24		N.D.*	
-3	58.08±20.64		94.52±2.19			
-4	51.11±18.36	-0.252	93.42±4.43	0.019		
-5	45.00±6.25	NS	91.10±3.27	NS		
L.casei						
0	53.51±6.24		60.71±8.20		70.04±7.71	
-3	47.62±10.53		69.45±6.54	0.245	78.37±6.87	0.302
-4	62.73±6.82	-0.384	66.52±10.24		74.75±5.79	NS
-5	53.50±6.40	NS	60.00±7.96	NS		
C.albicans						
0	63.42±12.01		82.06±3.32		86.40±3.39	
-3	75.20±15.20	0.366	87.12±3.90	0.581	93.64±2.57	0.314
-4	61.15±1.36	NS	82.02±2.17	P= 0.05	91.75±3.18	NS
-5	70.32±8.64		80.08±5.15			

Effect Of 10^{-3} M Fosfestrol On The Chemilumunescence Response Of Human PMN To S.aureus, S.pyogenes, E.coli, L.casei And C.albicans

S.aureus, S.pyogenes, E.coli, L.casei and C.albicans at 10^6 organisms per ml, as a stimulus for human PMN, gave similar patterns of chemiluminescence (CL) regardless of the organism used. Fosfestrol appears to inhibit CL from 0 to 30 minutes, and to stimulate CL from 30 to 60 minutes with C.albicans, L.casei and S.aureus as stimuli. It did not appear to affect the chemiluminescence of S.pyogenes- or E.coli-stimulated PMN, or unstimulated PMN. The results were reproducible and are represented graphically in fig 24.

Effect Of Fosfestrol On The Oxygen Consumption And Glucose Oxidation Of Latex-stimulated Human PMN

Fosfestrol at a concentration of 10^{-3} M stimulated glucose-1-^{14}C oxidation via the HMPS by 17.76%. This figure corresponds to a X^2 value of 9.64. The probability of this amount of stimulation occuring by chance is 2-5%, indicating that treatment of the opsonised, latex-stimulated PMN by 10^{-3} M fosfestrol significantly increases the amount of glucose oxidised to CO_2.

The oxygen consumption of fosfestrol-treated latex-stimulated PMN was inhibited by 18.22% and 15.91% over 30 and 60 minutes respectively. However on application of X^2 statistics,

FIGURE 24

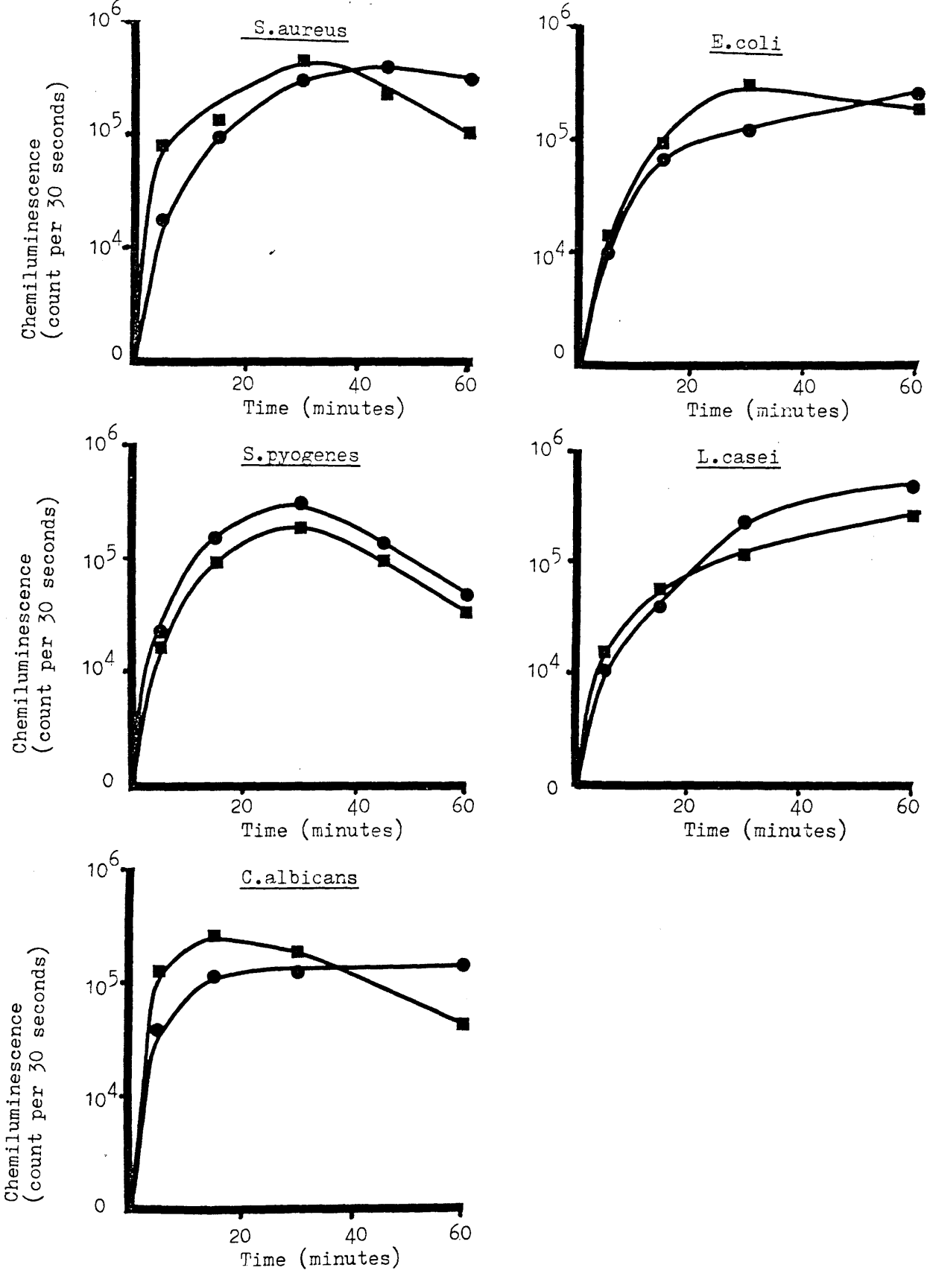
LEGEND

Chemiluminescence of PMN treated and untreated with 10^{-3} M fosfestrol and stimulated by S.aureus, E.coli, S.pyogenes, L.casei and C.albicans respectively.

■ - ■ Control PMN

● - ● 10^{-3} M fosfestrol-treated PMN

FIGURE 24


S.aureus
E.coli
S.pyogenes
L.casei
C.albicans
Chemiluminescence (count per 30 seconds)
Time (minutes)
10^6
10^5
10^4
0
20
40
60

values of 5.59 and 4.32 were obtained, these correspond to probabilities of 10-25%. Values of greater than 5% are deemed to be non-significant. The assay was performed three times and on two of these occasions, after 60 minutes the stimulated PMN suspension had a dissolved oxygen content of greater than 100%, the arbitrary value assigned to a suspension of human PMN at a concentration of 10^6 per ml. It was noted that on addition of the latex particles the amount of dissolved oxygen in the PMN suspension rose, however in the suspension containing fosfestrol the output of oxygen by the PMN was stimulated by 10-15%.

The Effect Of Fosfestrol On The Activities Of Lysozyme, Myeloperoxidase And Alkaline Phosphatase

Concentrations of fosfestrol of between 10^{-3} and 10^{-7} M had no significant effect on the activities of any of the tested enzymes.

The Effect Of Fosfestrol On The Release Of PMN Lysosomal Enzymes

Fosfestrol was found to have no significant effect on the release of lysosomal enzymes from stimulated human PMN.

The Effect Of Preincubation Of Fosfestrol At Various Concentrations With 0.3% Hydrogen Peroxide On Myeloperoxidase Activity

Lysosomal enzyme release and myeloperoxidase activity were found to be unaffected by fosfestrol which had been preincubated with 0.3% H_2O_2.

Electron Microscopical Examination Of C.albicans Within Fosfestrol Treated And Control Human PMN

After 30 minutes incubation, both control and fosfestrol-treated PMN were still intact. A number of PMN were without either engulfed or partially engulfed Candida cells, a valid determination of the proportion of these cells could not be calculated, as the number of sections screened did not allow for statistical analysis. Some PMN were in the process of phagocytosing Candida and exhibited extended pseudopodia around the yeast cells. The majority of PMN had engulfed one yeast cell and some had ingested two cells, fosfestrol did not appear to influence the rate or amount of phagocytosis after 30 minutes incubation.

There was little evidence in control and fosfestrol-treated preparations of intracellular degradation of the C.albicans.

Plates 3-17 show Candida ingested by both control and fosfestrol-treated PMN.

After 60 minutes incubation the majority of PMN surveyed were still intact, although some of the cells had started to aggregate. Of over 50 PMN examined, only two cells had not engulfed at least one Candida. There was a wide spectrum in the degree of degradation of the Candida cells within PMN. This variation in degradation could even be seen within a

single PMN. Plates 10, 15, 16. Differences in the type of vacuole enclosed the Candida were noted, some in loose and others in tight vacuoles. Plate 5. Fusion of granules with the phagocytic vacuole could be clearly seen, and extensive folding of the enclosing vacuole membrane was noted in a number of PMN. Plate 9.

Following 60 minutes incubation, some differences were observed in the morphology of the Candida within fosfestrol treated PMN.

There was some loss of integrity of the plasma membrane, and within some PMN it was seen to have detached from the yeast cell wall, this sort of degree of degradation was seen within only one of the control PMN. The cytoplasm of all the ingested Candida within both control and fosfestrol-treated PMN appeared to be less electron dense, there was a distinct lack of organelles within the Candida in most cases. However this intracellular deterioration had proceded still further in some fosfestrol-treated PMN, such that large 'empty' areas could be seen within the Candida. Plates 16 and 17. The distinct striations of the Candida cell wall were less visible within drug-treated PMN than within control PMN. In general the yeast cells within fosfestrol-treated PMN appeared to be more degraded than in control PMN, and the most deteriorated Candida were invariably found to be within fosfestrol-treated PMN, and the least degraded Candida within control PMN.

PLATE 1

Micrograph of a typical, unstimulated human PMN.

PLATE 2

Human PMN; note the typical multi-lobed nucleus.

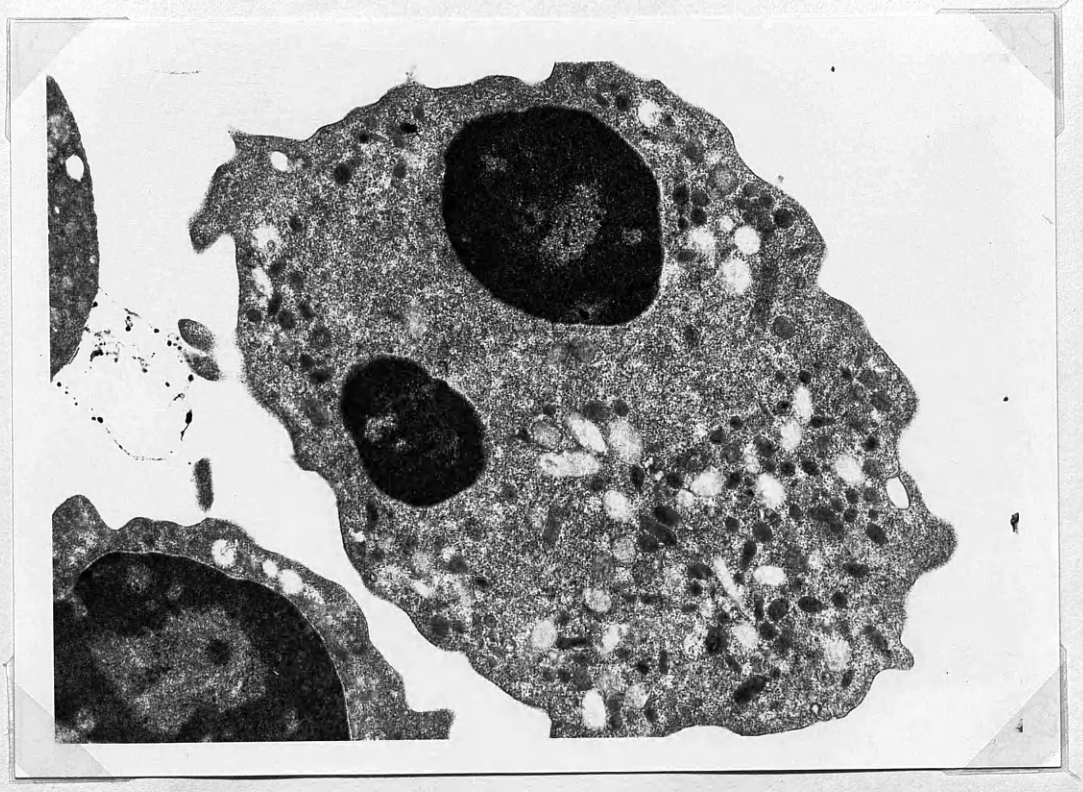


Plate 1

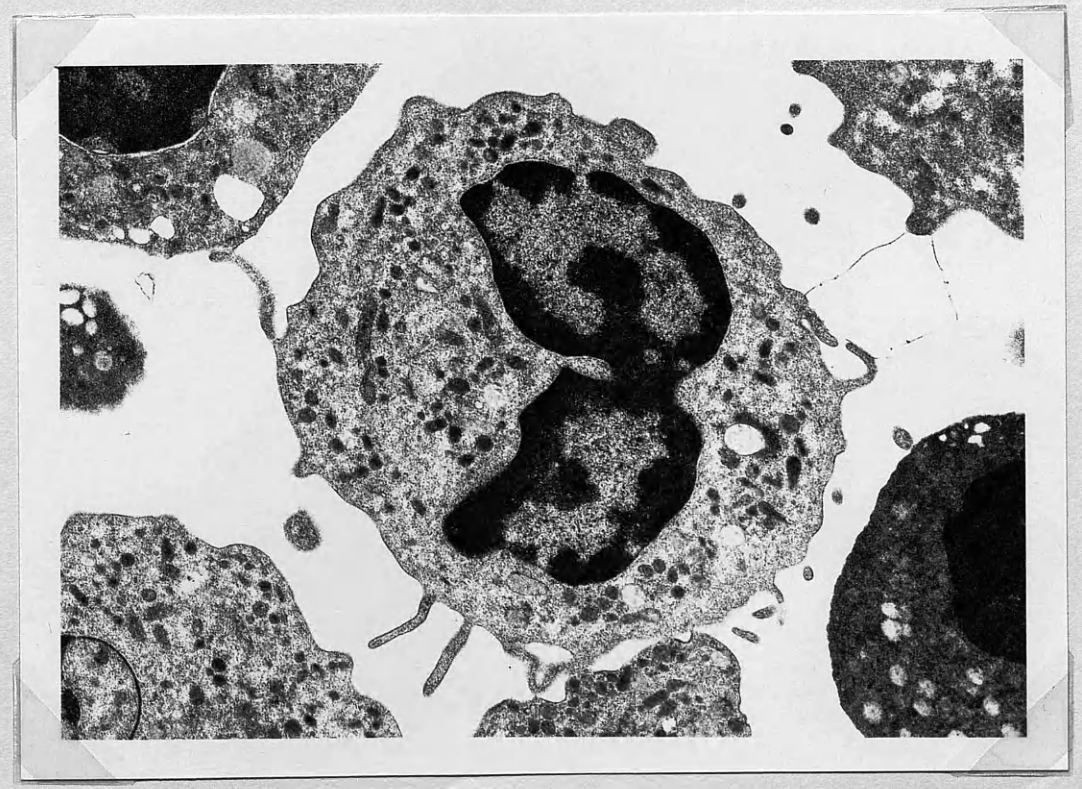


Plate 2

PLATE 3

Control PMN from after 30 minutes incubation with C.albicans. The PMN contains large intracellular granules. The ingested C.albicans does not show signs of degradation, the cytoplasm is electron-dense and striations on the cell wall are visible.

PLATE 4

Control PMN after 30 minutes incubation. The PMN has phagocytosed a budding yeast which contains inact intracellular organelles. The C.albicans are within "loose" vacuoles.

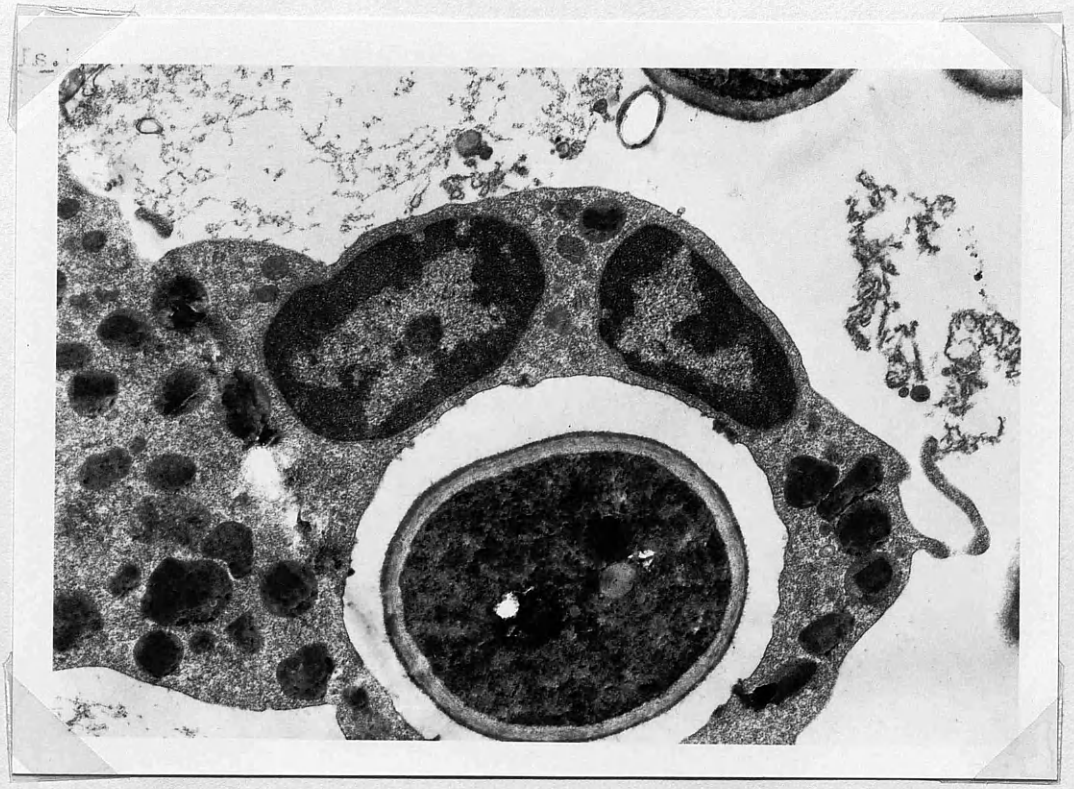


Plate 3

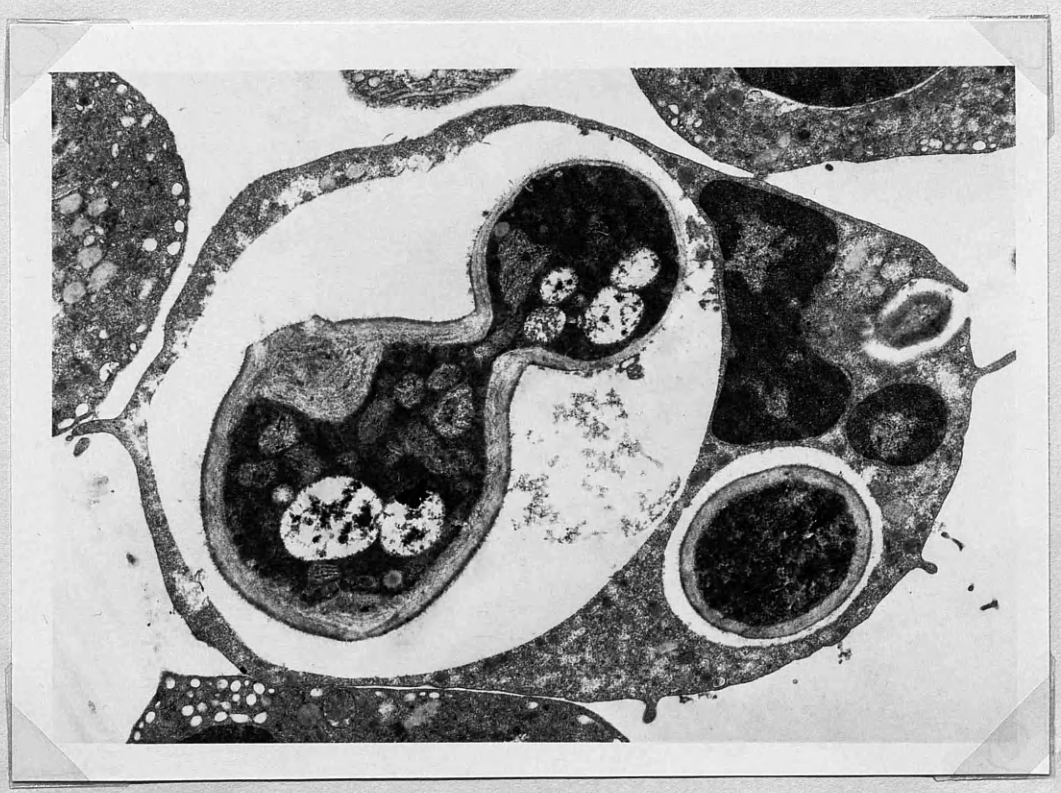


Plate 4

PLATE 5

Control PMN after 30 minutes incubation with C.albicans. The PMN contains two yeast cells, the larger cell is within a "tight" phagocytic vacuole. The PMN has a number of pseudopodia extended. Granules can be seen fused with the phagosome, where they release their contents.

PLATE 6

Fosfestrol-treated PMN after 30 minutes incubation with C.albicans.

The PMN contains an intact C.albicans cell, showing no signs of degradation.

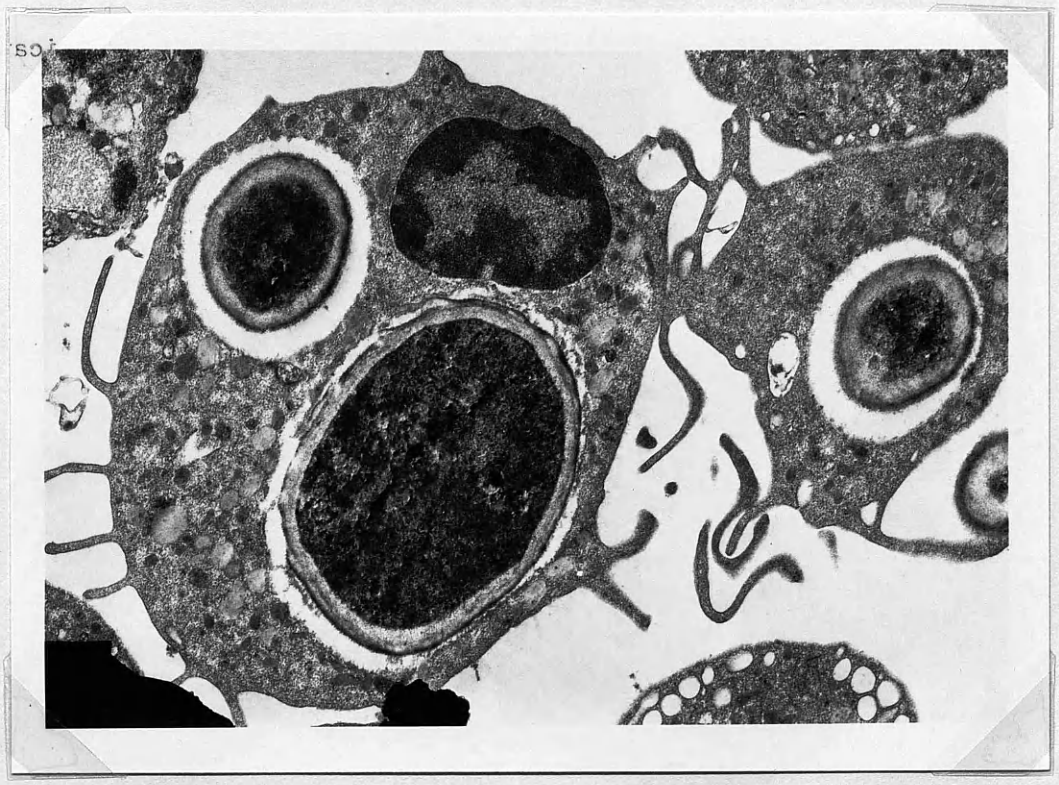


Plate 5

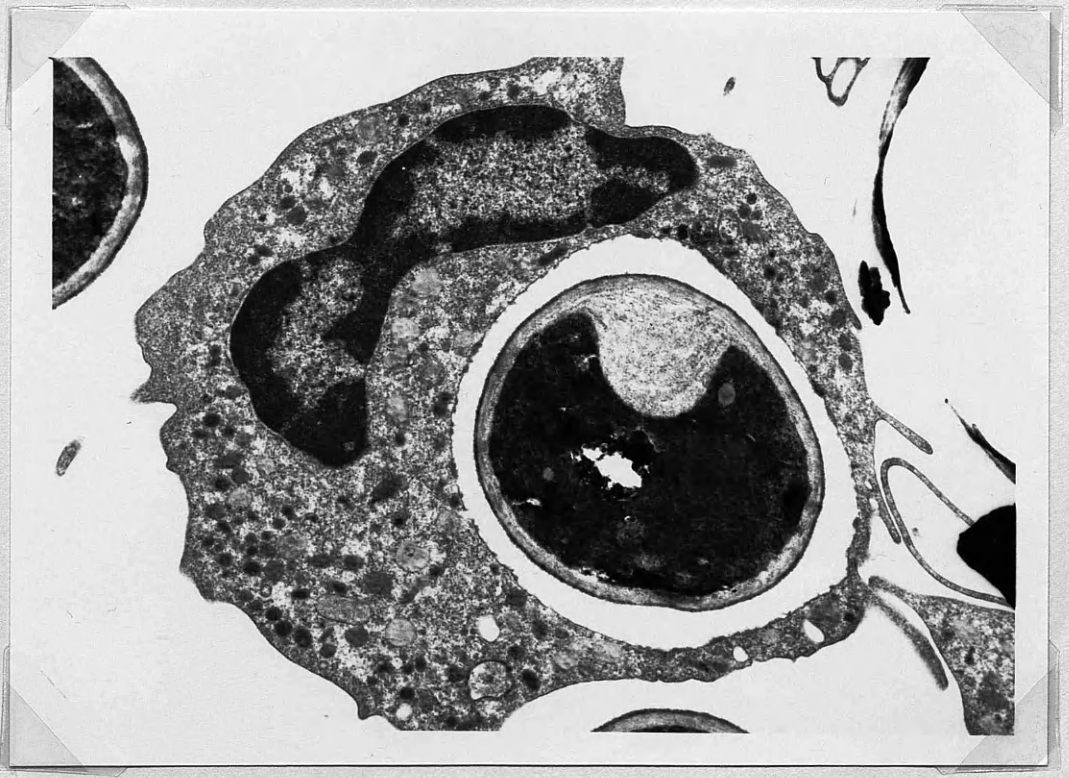


Plate 6

PLATE 7

Fosfestrol-treated PMN after 30 minutes incubation with C.albicans.

The ingested yeast cell appears undegraded.

PLATE 8

Fosfestrol-treated PMN after 30 minutes incubation with C.albicans.

The yeast cells contain chromatin which is very hard and sometimes causes tears when sections are cut through it, as seen here.

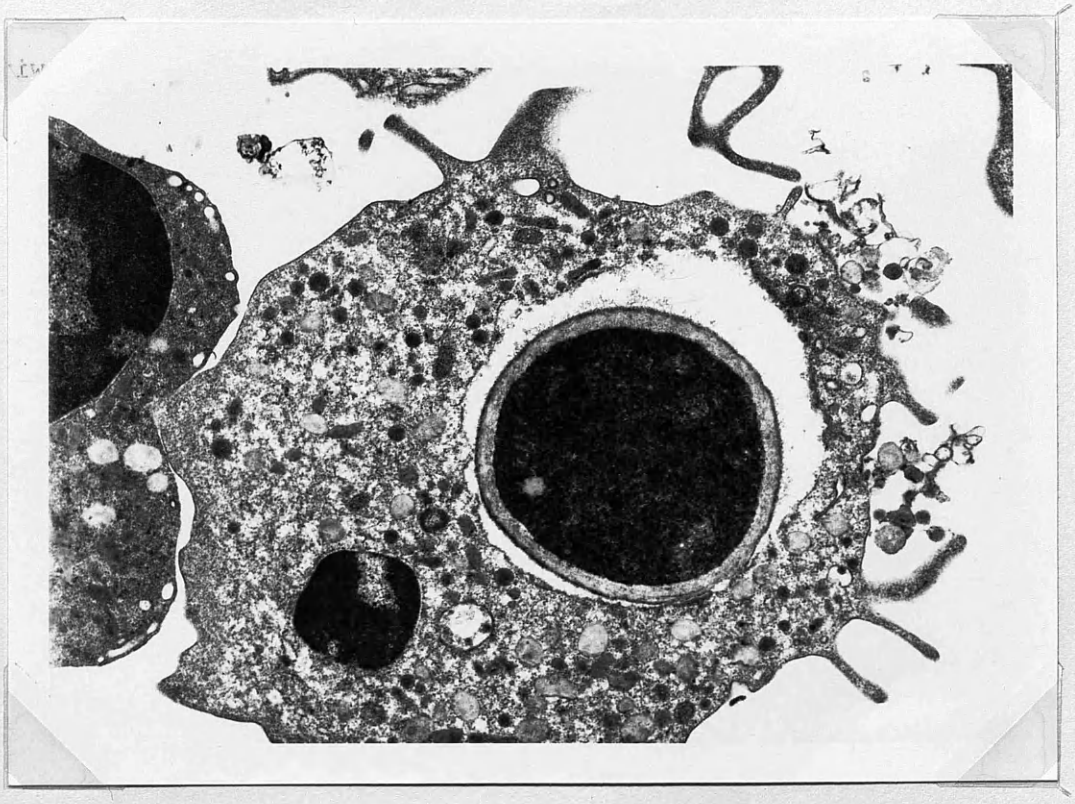


Plate 7

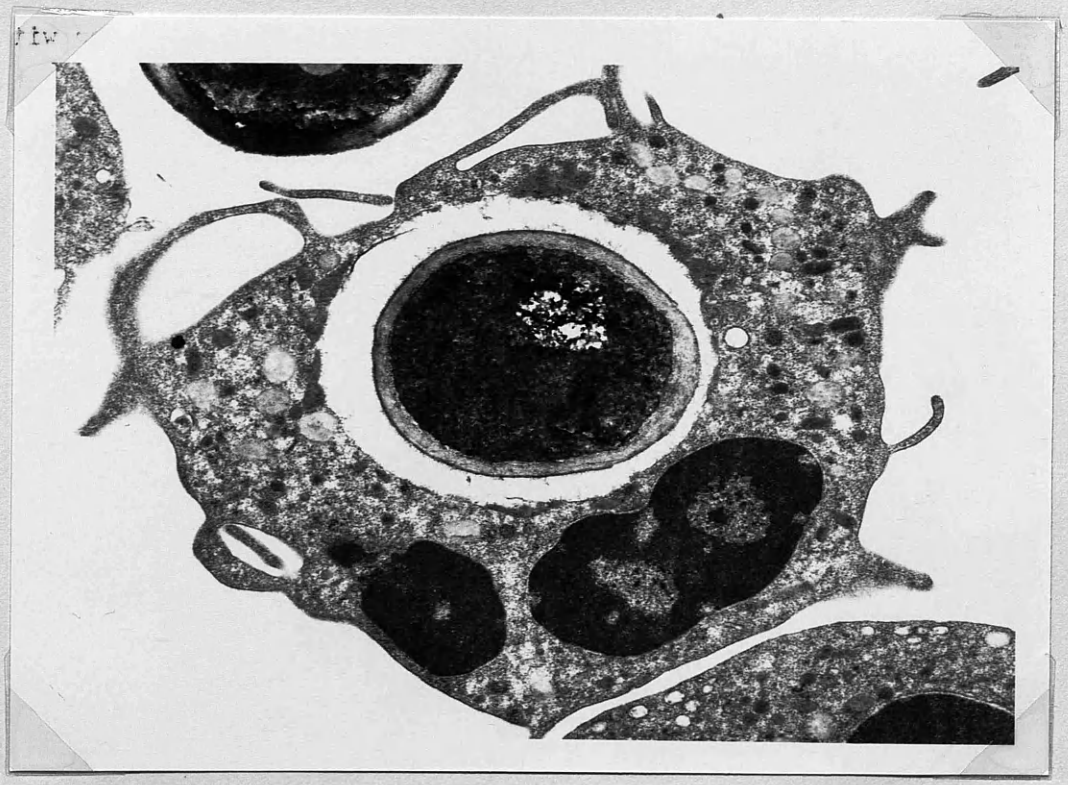


Plate 8

PLATE 9

Control PMN after 60 minutes incubation with C.albicans. The yeast cell wall does not appear to have such sharply defined striations.

PLATE 10

Fosfestrol-treated PMN after 30 minutes incubation with C.albicans.

Four yeast cells have been ingested and show differences in their stage of degradation.

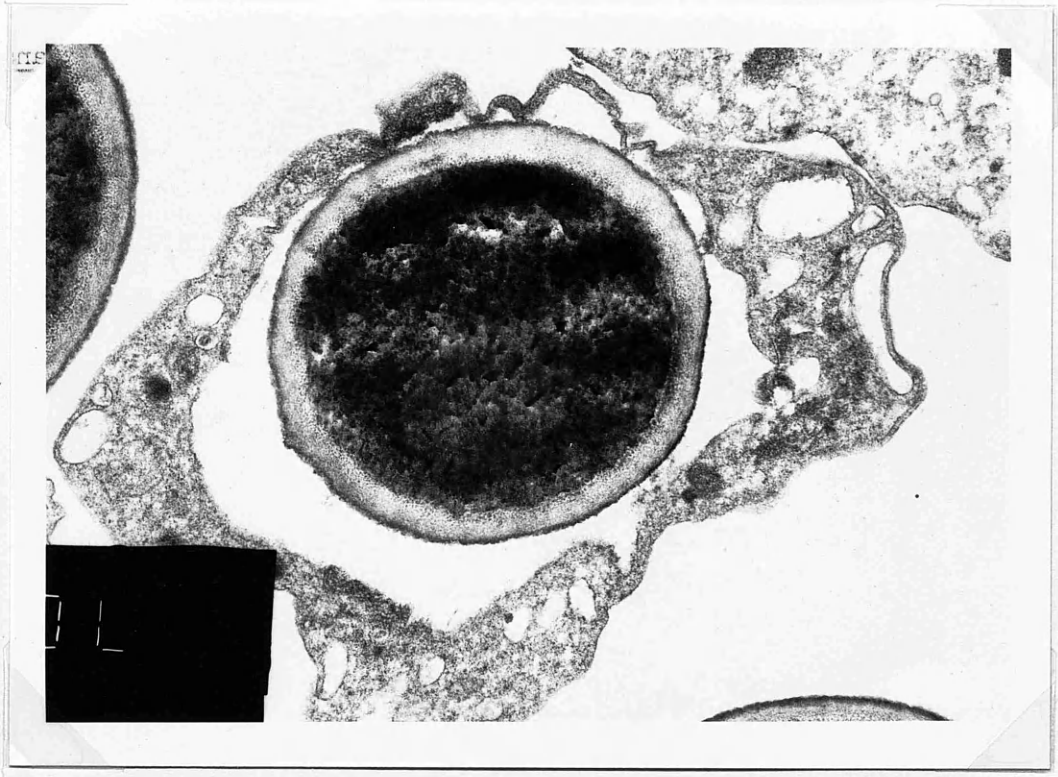


Plate 9

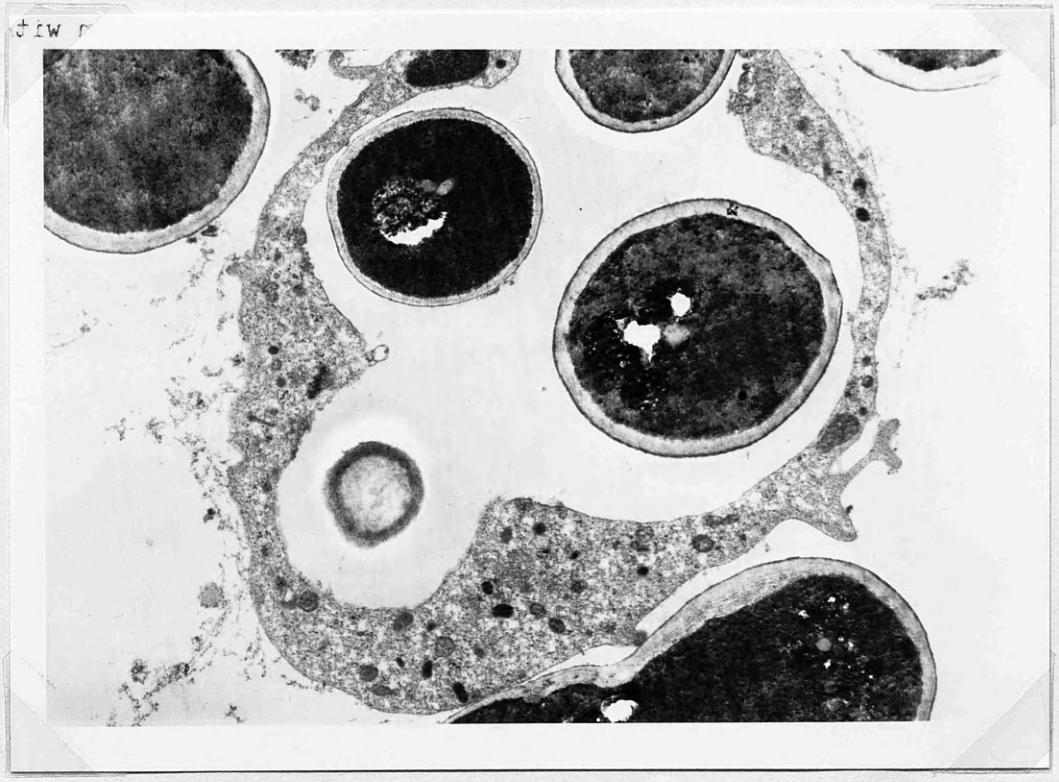


Plate 10

PLATE 11

Control PMN after 60 minutes incubation with C.albicans.

PLATE 12

Fosfestrol-treated PMN after 60 minutes incubation with C.albicans.

The yeast is enclosed within the PMN by a very thin membrane.

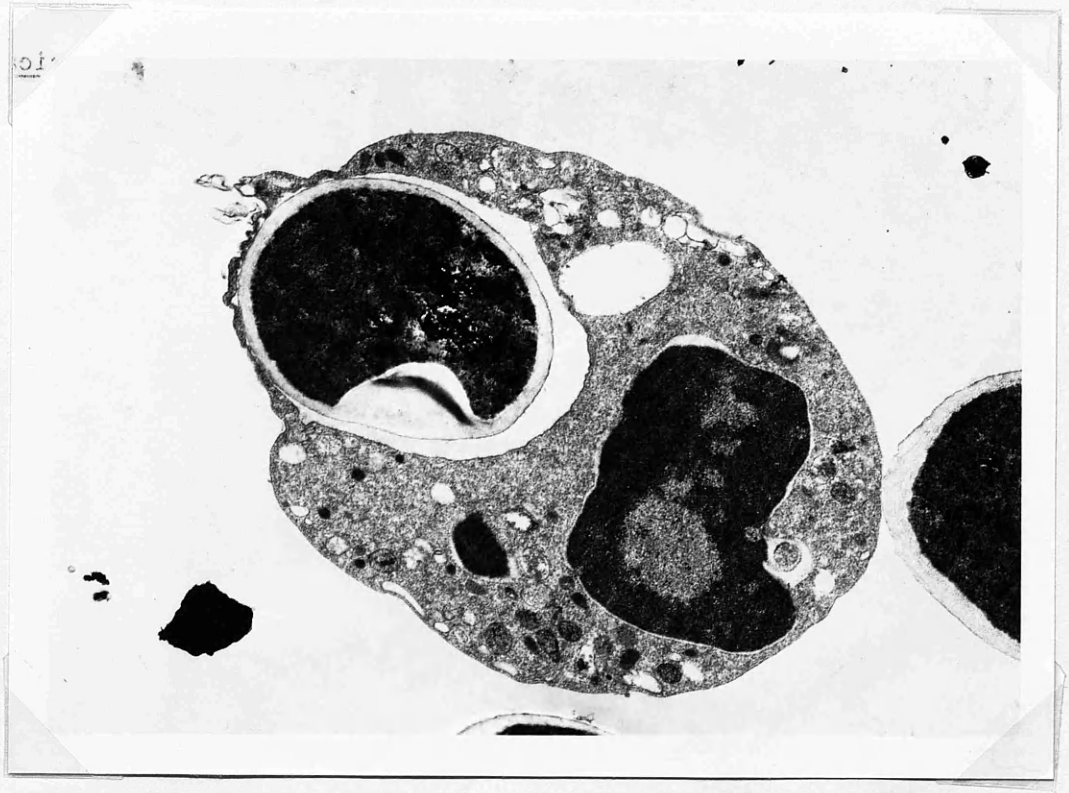


Plate 11

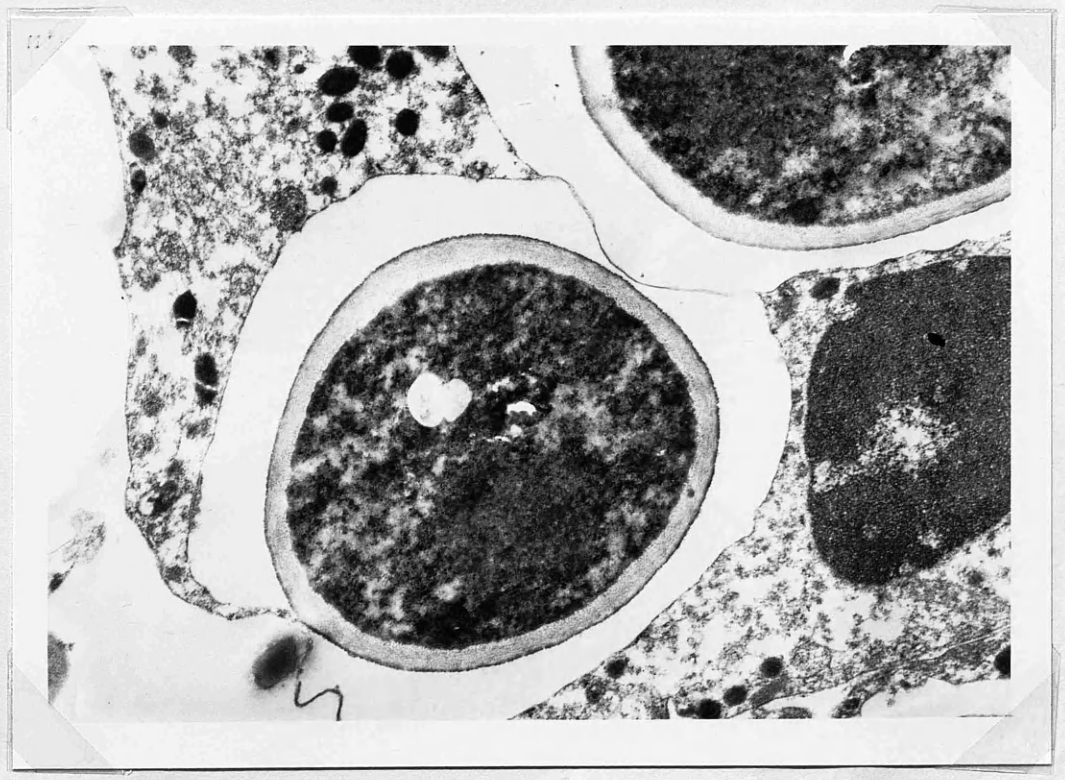


Plate 12

PLATE 13

Fosfestrol-treated PMN after 60 minutes incubation with C.albicans.

The membrane enclosing the phagocytosed yeast cells is very thin. The C.albicans show little evidence of degradation.

PLATE 14

Fosfestrol-treated PMN after 60 minutes incubation with C.albicans.

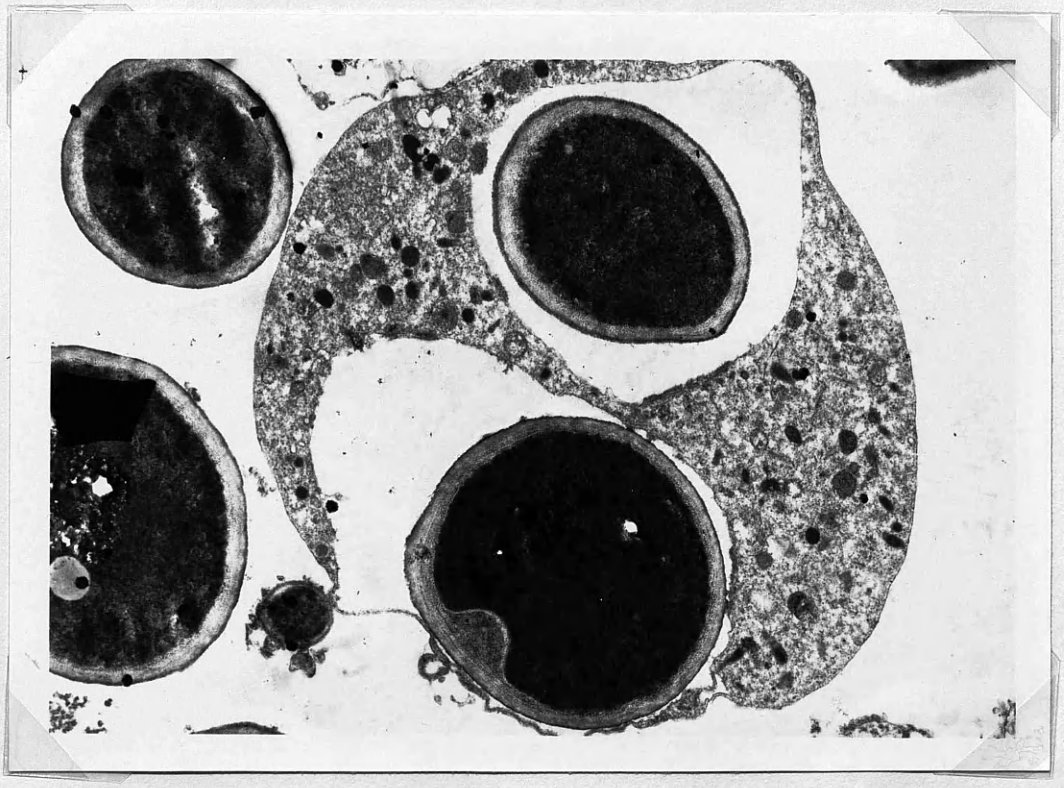


Plate 13

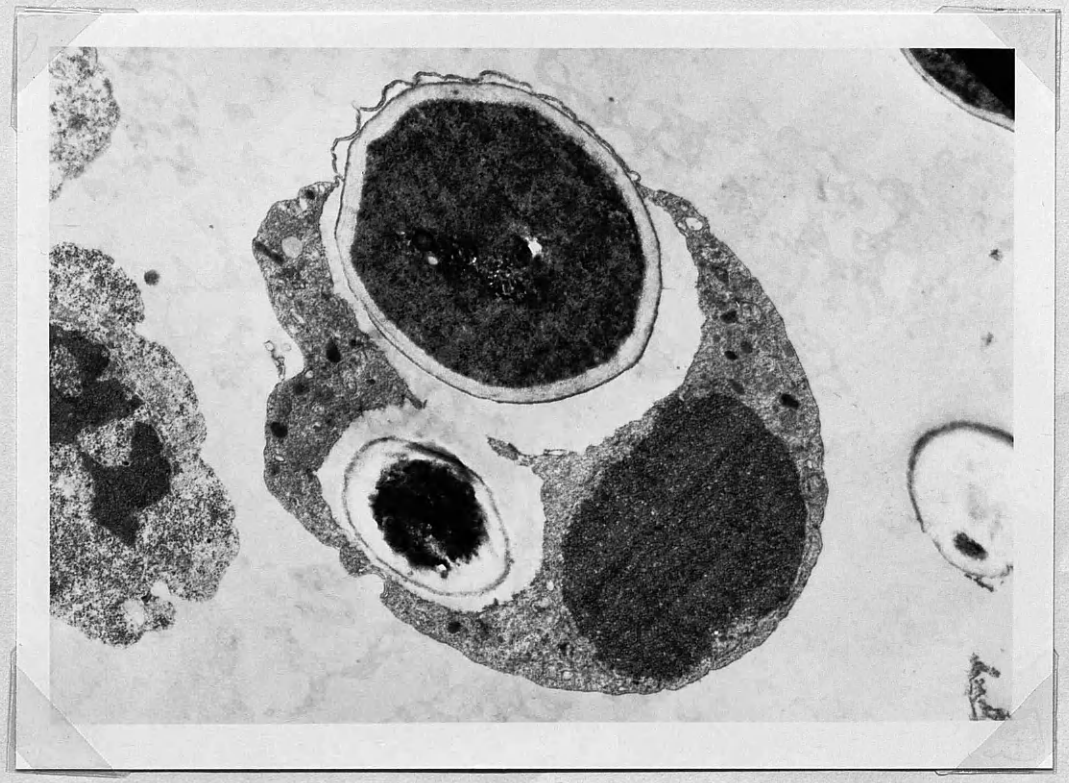


Plate 14

PLATE 15

Control PMN after 60 minutes incubation with C.albicans.

PLATE 16

Fosfestrol-treated PMN after 60 minutes incubation with C.albicans.

The PMN contains one cell showing some signs of degradation, and an extensively degraded yeast; the cell membrane has detached from the cell wall.

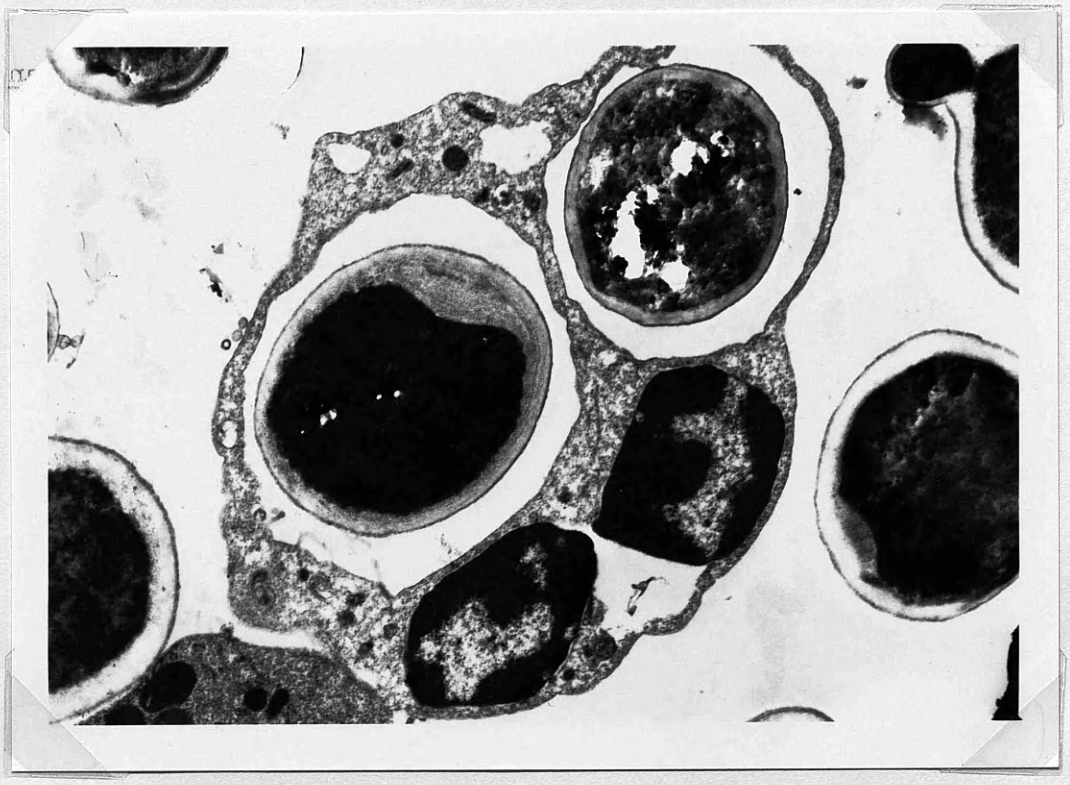


Plate 15

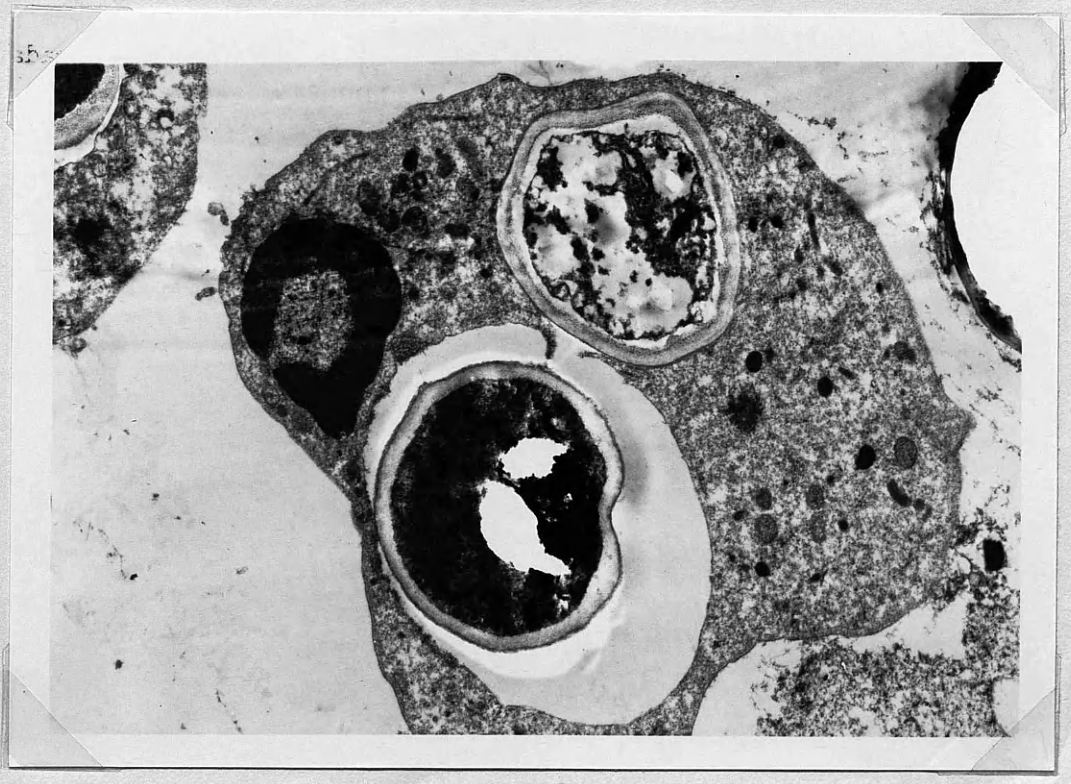


Plate 16

PLATE 17

Fosfestrol-treated PMN after 60 minutes incubation with C.albicans.

The yeast cell within the PMN is held in a "tight" vacuole, the cell wall has lost its rigidity, the plasma membrane has detached and the cytoplasm containing the cell's organelles has become "patchy", indicating extensive degradation.

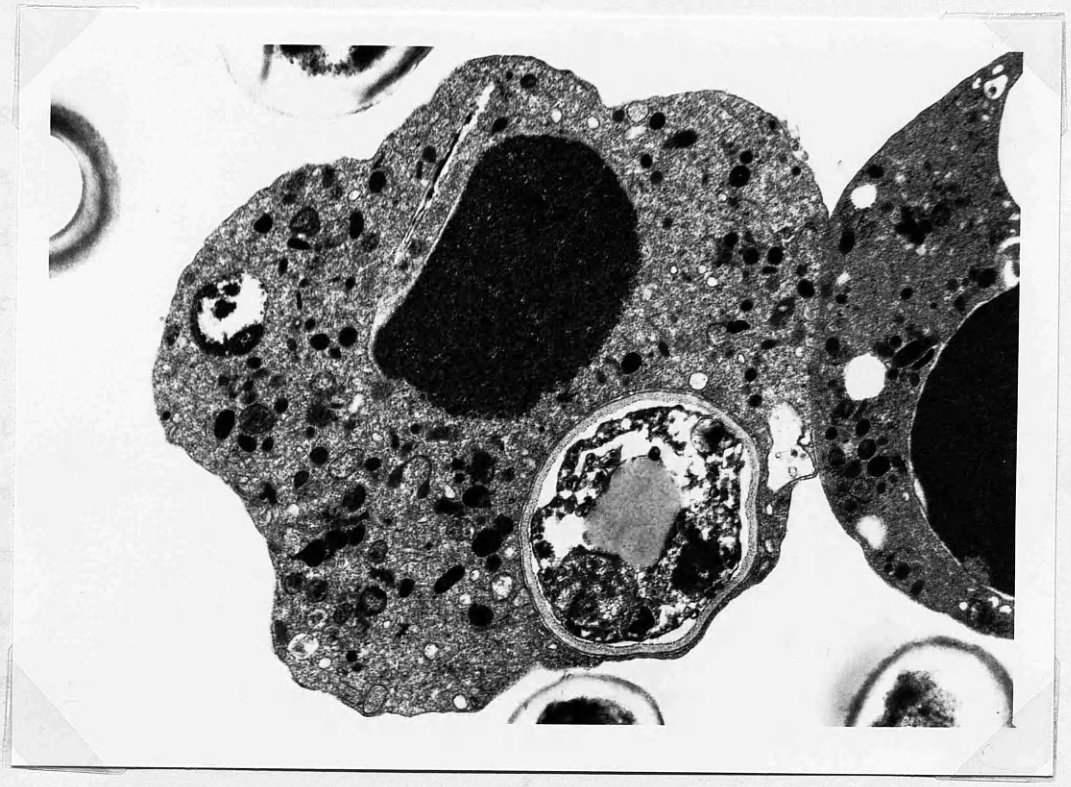


Plate 17

7.3 Discussion.

During pregnancy, total oestrogen of plasma increase, this is accompanied by a greater ability to combat certain infections, and an increased susceptibility to others, such as urinary tract infections. The total white blood cell count in peripheral blood rises and this change is mainly accountable to PMN and monocytes. The PMN of pregnant females exhibit hyper-phagocytic and bactericidal activities (Mitchell *et al*, 1965, 1966). The stimulation of PMN activity during pregnancy has been attributed by some authors to oestrogens, although progesterone and peptides cannot be discounted. Fosfestrol is a water soluble oestrogen compound, and as such can be used at concentrations as high as 10^{-3} M, which are impossible to reach *in vitro* in aqueous solution with steroid oestrogens.

In this study selected parameters of PMN function were examined for their response to treatment with fosfestrol. Chemotaxis and random movement were found to be unchanged, as was the phagocytosis of *S.aureus*, *S.pyogenes*, *E.coli*, *L.casei* and *C.albicans*. However the PMNs' ability to kill these organisms was affected. After 30 minutes incubation, fosfestrol at a concentration of 10^{-3} M inhibited the killing of *S.aureus*, *S.pyogenes* and *L.casei*, however the results did not show significant dose-related inhibition, except in the case of *S.aureus* ($P=0.05$). *E.coli* and *C.albicans* were apparently killed more efficiently, but the results did not

reach statistical significance. After 60 minutes incubation, there was no significant difference between control and fosfestrol treated PMNs' killing of the bacteria, but microbicidal activity against C.albicans was increased and was significantly dose-related. E.coli and L.casei also appeared to be killed more efficiently.

Thus it would seem that fosfestrol stimulates the PMN to kill E.coli and C.albicans more efficiently from 30 minutes onward, L.casei from 60 minutes onward, S.pyogenes only after 120 minutes and S.aureus not at all. The results did not reach significance, but there appears to be a general pattern of inhibition followed by stimulation. The chemiluminescence studies showed a similar pattern of inhibition followed by stimulation.

The oxygen consumption of fosfestrol-treated PMN was not significantly different from control cells, however it was interesting to note that the initial response of the PMN to the opsonised latex particles was greater than in control PMN. This large output of oxygen species may have masked the amount of oxygen consumed by the PMN. This result appears to conflict with the evidence from the chemiluminescence results of decreased production of reactive oxygen species, however it is possible that fosfestrol somehow quenches the amount of chemiluminescence detected, as it was not possible to establish adequate controls to cover the

possibility of fosfestrol interfering with the detection of chemiluminescence. However it is interesting to note the parallel with results from the microbicidal assays.

HMPS activity was stimulated over a period of two hours incubation, this suggests that more H_2O_2 would be available in the PMN to take part in the killing of micro-organisms via the MPO-H_2O_2-halide system or in non-enzymatic ways such as by reacting to form hydroxyl radicals (Repine et al, 1981).

Neutrophil peroxidase activity in women during the perinatal period has been found to increase (Sulowicz et al, 1981). Jacobs et al (1973) and Selvaraj et al (1980) have suggested that oestrogens stimulate MPO- mediated amino acid decarboxylation, although they did not observe stimulation of guaiacol oxidation. They proposed that oestrogens influence the MPO-H_2O_2-halide system, stimulating the first step of the decarboxylation reaction, chloramine formation, possibly by altering the affinity of the enzyme for Cl^- ions. However, their results may be questionable due to the limits of solubility of steroid compounds. They used putative concentrations of up to 10 μg/ml, equivalent to a concentration of around 10^{-4} M, and even using absolute ethanol as diluent, this seems very much higher than the 10^{-7} M limit of aqueous solubility. Fosfestrol at concentrations of between 10^{-3} and 10^{-7} M had no significant effect on MPO activity as measured in this study.

It is known that oestrogens are a substrate for, and can be altered by, peroxidases (Klebanoff and Clark, 1978). During phagocytosis, oestrogen is converted to an "alcohol-precipitable" form by PMN, and can be visualised autoradio-graphically, bound to the cells ie. it is protein bound. Oestrogens are 'inactivated' by peroxidase and H_2O_2 and can stimulate certain reactions catalysed by peroxidase of plant origin, raising the possibility that the interaction of oestrogens and PMN may influence the turnover of oestrogen and neutrophil function (Klebanoff and Segal, 1960). The interaction of oestrogens with peroxidase results in the formation of catecholoestrogens (Ball and Knuppen, 1980). Thus although fosfestrol did not significantly affect MPO activity as assayed in this study, it is possible that the compound, on contact with products of the respiratory burst, becomes chemically altered, and 'activated', accounting for the observed stimulation of the PMNs' microbicidal function. The reactive oxygen species generated during the respiratory burst include H_2O_2, O_2^-, $OH^\bullet$ and O_2^1, these compounds are known to be capable of transferring their electronic energy to acceptor molecules or use it to oxidise target molecules. Radical initiated processes are particularly deleterious because being conservative and propogative, they can produce secondary and tertiarary free radicals derived from lipids, amino acids, glutathione, ascorbic acid and components of nucleic acids (Trush et al, 1982). Superoxide from xanthine oxidase can convert 2-hydroxyoestrogens to reactive intermediates, possibly

quinones and/or semi-quinones capable of covalently binding protein. In addition, radicals, quinones and/or semi-quinones which are intermediates in the oxidation of diethylstilboestrol (DES) by peroxidases have been implicated in the toxicity of DES to the hamster kidney and mouse uterus (Mezler and McLachlan, 1978; Klicka et al, 1983). However on preincubation with H_2O_2, fosfestrol still failed to significantly alter MPO activity, therefore possibly one or more of the other respiratory burst products alters the compound.

Lysozyme and alkaline phosphatase are involved in the cidal and degradative function of the PMN and their activity was unaffected by fosfestrol, however there has been a report of stimulated alkaline phosphatase activity in PMN under the influence of stilboestrol (Moszczynski, 1976). Neutrophil alkaline phosphatase activity also increases in the second half of the menstrual cycle (Radwanska et al, 1971). Fosfestrol-stimulation of these enzymes may also occur through a mechanism similar to that proposed for MPO.

C.albicans was chosen for more extensive study, as fosfestrol-treated PMN killed this organism more efficiently than control cells. Also, as an eukaryotic organism, its structure is more complex, it contains organelles whose integrity might be affected by the degradative enzymes of the PMN. Early investigations into the ability of human PMN to kill ingested C.albicans in vitro indicated some failure in intracellular

killing following ingestion of the organisms. This was due to up to 64% of the PMN being lysed by germ-tube production by the Candida (Leijh et al, 1977). In the present study germ-tube formation on exposure to serum has been prevented by incubating the Candida for five days at a temperature of 30°C, as recommended by Leijh et al, (1977). Despite this, considerable variation in the percentage of ingested yeast cells killed by human and animal PMN has been reported, ranging from 18.5 to 75% (Rogers and Balish, 1980). The figures obtained in this study indicate that up to 80% of ingested Candida may be killed within 60 minutes. However, on treatment with 10^{-3} M fosfestrol, this figure rose significantly to 87%. Fosfestrol in aqueous solution has a pH of 7.2-7.5, and as such would not be expected to exert a pH effect on any of the degradative enzymes of the PMN.

On electron microscopical examination, it was found that fosfestrol, did not appear to affect phagocytosis, although after 60 minutes incubation fosfestrol-treated PMN contained more extensively degraded Candida cells. After treatment with lysosomal enzyme extracts, morphologic alterations are produced in the cells, such as an increase in granularity and vacuolation of the yeast's cytoplasm (Lehrer, 1969). However, Taschdjian et al (1971) using an anti-Candida fluorescent test, concluded that the outermost layers of the cell wall were the initial targets in the process of intracellular destruction. It is well recognised that fixation is a key factor in the ultra-

structural demonstration of fungal cell wall detail, and in electron microscopical studies this may contribute to the lack of definition of intracellular organisms. However in the sections examined extracellular organisms' cell walls appeared to be intact, showing defined striations and a dense cytoplasm, therefore the effects of the fixatives used in this study appear to have been minimal.

Incubation times of greater than 60 minutes were not used, as after this time the PMN tended to form aggregates. It was interesting to note the different types of phagolysosomes formed by the PMN around the Candida, some within tight and others within loose vacuoles: the more extensively degraded organisms were generally within tight vacuoles. Cech and Lehrer (1984) described unsealed- and sealed vacuoles, they proposed that approximately 40% of the total phagolysosomes formed "maintain functional communication to the cell's exterior" and as such support antimicrobial processes substantially less well than do completely sealed ones. During phagocytosis the PMN's plasma membrane is known to undergo substantial remodelling, if fosfestrol affected the rate of acylation of lysophosphatides, a higher proportion of sealed vacuoles could result, however there is no evidence to support this idea, but it could in part account for the stimulatory effect of fosfestrol on intracellular killing by PMN.

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